# Mechanistic and synergistic actions of the *in vitro* antimycobacterial and immunomodulatory properties of *Knowltonia vesicatoria* (L.f.) Sims

by

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## Abstract

Three South-African plant extracts, *Knowltonia vesicatoria* (L.f.) Sims (aerial, ethanol), *Pelargonium sidoides* DC. (root, ethanol) and *Euclea natalensis* A.DC. (root, chloroform) were subjected to *in vitro* antimycobacterial, cytotoxicity, intracellular antimycobacterial and immune modulating assays to investigate the possibility of plant therapies that can serve as a treatment for the depleting immune system and the virulence of *M. tb* in TB/HIV-coinfected sufferers. A Glutathione reductase enzyme (Gtr) assay was employed as a surrogate for the *M. tb* enzyme (Mycothione reductase) to screen samples for selective enzyme inhibition as the possible mechanism of action. The same set of assays was undertaken for synergistic amalgamations of the three extracts in combination with the first line drug, isoniazid (INH).

Extracellular antimycobacterial tests included the radiometric BACTEC method and a colorimetric microplate dilution assay. Sample cytotoxicity on human macrophages (differentiated U937 cells) was determined using a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide (XTT) proliferation kit. Intracellular activity was established by enumeratring the colony forming units (CFU) via the colony counting system ColorQCount. To determine the immunological effects of samples on the macrophages, cytokine profiles were analyzed using cytometric bead array technology.

To eliminate the possibility of tannins as the antimycobacterial actives in the *K. vesicatoria* extract, the extract was subjected to a solvent partitioning method to remove the polyphenols. It was confirmed that the tannin-free fraction had poor antimycobacterial activity when tested against *M. smegmatis* exhibiting a MIC of 2500 µg/mL and was not active against *M. tb* at the highest concentration tested. The activity of the whole *Knowltonia* extract remained superior to the

fractions obtained by solvent partitioning, with a MIC of 625.0 µg/mL against *M. smegmatis* and a MIC of 50.00 µg/mL against *M. tb*.

It was found that the synergistic combination of *K. vesicatoria* with INH exhibited the best fractional inhibitory concentration (FIC) of 0.25 and a fifty percent inhibitory concentration ($IC_{50}$) of 121.0 µg/mL on the macrophages, indicating a therapeutic index (TI) of 19.00. Two compounds, a sterol (*stigmasta-5,23-dien-3-ol*) and a lactone (*5-(hydroxymethyl)furan-2(5H)-one*) were successfully isolated from *K. vesicatoria*, as the first report of isolation for both compounds from this plant. The antimycobacterial properties of these compounds have not been reported earlier. The sterol was the most active sample against a drug-sensitive strain of *M. tb* with a minimum inhibitory concentration (MIC) of 50.00 µg/mL. The extract of *K. vesicatoria* was tested against two drug resistant strains of *M. tb* (resistant to INH, ethambutol (EMB), streptomycin (STR) and rifampicin; RIF) which resulted in a MIC of 50.00 µg/mL on both strains, indicating a mechanism of action different to INH, EMB, STR and RIF. The isolated lactone revealed 70% inhibition of Gtr. The only samples that exhibited any significant intracellular antimycobacterial activity were the crude extracts of *K. vesicatoria* and *E. natalensis* with 80 and 60% *M. tb* growth inhibition at concentrations of 50.00 and 8.00 µg/mL, respectively.

Immunological activity of selected test samples was determined by measuring cytokine levels (IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α) of TB-infected and uninfected macrophages. A slight Th1-type response was seen for infected cells treated with 7-methyljuglone and *P. sidoides* at their $IC_{50}$ values of 18.00 µg/mL and 43.00 µg/mL, respectively. Further studies are required to confirm the immunological activities by evaluating various other cytokines such as IL-12.

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### Chapter 2.

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## LIST OF ABBREVIATIONS

**$^{14}CO_2$:** Carbon 14-labeled carbon dioxide
**2D:** Two dimensional
**7-MJ:** 7-methyljuglone
**Ab:** antibody
**$AgNO_3$:** silver nitrate
**AIDS:** acquired immunodeficiency syndrome
**APC:** antigen presenting cells
**ATCC:** American Type Culture Collection
**BCG:** bacille Calmette-Guérin
**BCGF-II:** B cell growth factor II
**BPI:** bactericidal/permeability-increasing protein
**CAM:** complementary and alternative medicine
**CBA:** cytometric bead array
**CC:** column chromatography
**CD:** cluster of differentiation
**CD4+:** cluster of differentiation 4 glycoprotein
**CD8+:** cluster of differentiation 8 glycoprotein

**CDC:** Centres for Disease Control and Prevention

**CFU:** colony forming units

**$CHCl_3$:** chloroform

**CMI:** cell mediated immunity

**COSY:** correlation spectroscopy

**CR:** complement receptor

**CSF:** colony stimulating factor

**CSIF:** cytokine synthesis inhibitory factor

**$dH_2O$:** Distilled water

**DMSO:** Dimethyl sulfoxide ($C_2H_6OS$)

**DNA:** deoxyribonucleic acid

**DOTS:** directly observed therapy short-course

**DTH:** delayed type hypersensitivity

**DTNB:** Dithionitrobenzoic acid or Ellman's reagent

**EDTA:** ethylenediaminetetraacetic acid

**ELISA:** enzyme-linked immunosorbent assay

**EMB:** ethambutol

**EPs**®: Extract of *Pelargonium sidoides* (a registered preparation)

**ETH:** ethionamide

**EtOAc:** ethylacetate

**EtOH:** Ethanol

**Eq:** equation

**FAD:** flavin adenine dinucleotide

**Fc:** crystallizable fraction of the Ig molecule

**FCS:** Foetal calf serum

**FDA:** Food and Drug Administration

**FDR:** flavoprotein disulfide reductase

**$FeCl_3$:** Ferric chloride

**FIC:** fractional inhibitory concentration

**G+C:** guanine plus cytosine

**GFP:** green fluorescent protein

**GI:** growth index

**GL:** glycolipids

**GM:** granulocyte-macrophage

**GSH:** Glutathiol or glutathione (reduced)

**GSSG:** Glutathione (oxidised) or Glutathione disulfide

**Gtr:** Glutathione disulfide reductase

**HEPES:** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

**HIV:** human immunodeficiency virus

**HMBC:** Heteronuclear Multiple Bond Coherence

**HMQC:** Heteronuclear Multiple Quantum Coherence

**HNP:** human neutrophil peptides

**$IC_{50}$:** fifty percent inhibitory concentration

**IFN:** interferon

**Ig:** immunoglobulin

**IL:** interleukin

**INH:** isoniazid

**iNO:** inorganic nitric oxide

**iNOS:** inducible nitric oxide synthase

**INT:** *p*-iodonitrotetrazolium chloride

**kDa:** kiloDalton

**Km:** Michaelis constant

**LAM:** lipoarabinomannan

**LC:** liquid chromatography

**LipDH:** lipoamide dehydrogenase

**LJ:** Löwenstein-Jensen

**LPS:** lipopolysaccharide

**LSP:** large sequence polymorphism

***M. tb*:** *Mycobacterium tuberculosis*

**MABA:** microdilution alamar blue assay

**MBC:** minimum bactericidal concentration

**MDP**: muramyl dipeptide

**MDR:** multiple drug-resistant

**MeOH:** Methanol

**MGIT:** Mycobacteria Growth Indicator Tube

**MHC:** major histocompatibility complex

**MIC:** minimal inhibitory concentration

**MIF:** methanol-insoluble fraction

**MOI:** multiplicity of infection

**MOTT:** mycobacteria other than tuberculosis

**MR:** mannose receptors

**MRA:** microdilution resazurin assay

**MRC:** Medical Research Council

**MS:** mass spectrometry

**MSH:** mycothiol or mycothione (reduced)

**MSSM:** mycothione (oxidised) or mycothiol disulfide

**MTC:** *Mycobacterium tuberculosis* complex

**Mtr:** mycothiol disulfide reductase or mycothione reductase

**MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

**NaCl:** Sodium chloride

**NADPH:** nicotinamide adenine dinucleotide phosphate

**NAPPO:** North American Plant Protection Organisation

**NBT:** Nitro blue tretrazolyum

**NFκB:** Nuclear Factor kappa B

**NK:** natural killer

**NMR:** Nuclear magnetic resonance

**NO:** nitric oxide

**NOESY:** nuclear Overhauser effect spectroscopy

**NQ:** naphthoquinone

**OADC:** oleic acid, albumin, dextrose, catalase

**OD:** optical density

**PA:** pyranizamide

**PANTA:** polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin

**PAS:** p-aminosalicylic acid

**PBS:** phosphate buffered saline

**PCR:** polymerase chain reaction

**PE:** phycoerythrin

**PGE:** prostaglandin E

**PKC:** Protein Kinase C

**PMA:** phorbol 12-myristate 13-acetate

**PPD:** purified protein derivative

**PRU:** H.G.W.J Schweicherdt Herbarium, University of Pretoria

**RIF:** rifampicin

**RPA:** ribonuclease protection assay

**RPMI:** Roswell Park Memorial Institute

**SD:** standard deviation

**SDS:** sodium dodecyl sulphate

**SOD:** superoxide dismutase

**sp.:** species

**ST:** sulfatides

**STR:** streptomycin

**TAP:** transporter associated with antigen processing

**TB:** tuberculosis

**TEM:** Transmission Electron Microscopy

**TGF:** transforming growth factor

**Th0:** Naive T helper lymphocyte

**Th1:** T helper 1 lymphocyte

**Th2:** T helper 2 lymphocyte

**TLC:** thin-layer chromatography

**TLR:** Toll-like receptor

**TM:** traditional medicine

**TNF:** tumour necrosis factor

**TPA:** 12-o-tetradecanoyl 13-acetate

**TRF:** T cell replacing factor

**Trxr:** thioredoxin reductase

**Ttr:** trypanothione reductase

**USAID:** United States Agency for International Development

**UV:** Ultra violet

**Vis:** Visual

**Vmax:** maximum velocity

**WHO:** World Health Organisation

**XDR:** extensively drug resistant

**XTT:** 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide

**ZN:** Ziehl-Neelsen

# CHAPTER 1
# INTRODUCTION and LITERATURE REVIEW

## 1.1. Introduction

### *1.1.1. History of Tuberculosis*

*Mycobacterium tuberculosis* (*M. tb*), the aetiological agent of tuberculosis (TB), is a highly successful human pathogen that kills nearly 3 million individuals each year. *Mycobacterium tuberculosis* has been coevolving with its human host much longer than previously thought (Gutierrez *et al*., 2005). It is believed that the members of the *Mycobacterium tuberculosis* complex (MTC; *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. pinnipedii*, *M*. *avium*, *M*. *intracellulare*, *M*. *scrofulaceum* and *M. caprae* species) are the clonal progeny of a single successful ancestor, resulting from a recent evolutionary bottleneck that occurred 20,000 to 35,000 years ago (Brosch *et al*., 2002; Gutacker *et al* 2002; Hughes *et al*., 2002; Sreevatsan *et al*., 1997). The ancestral progenitor species is estimated to be as old as 3 million years. This suggests that our remote hominid ancestors may well have already suffered from tuberculosis. Recent findings from genetic data open novel perspectives for unravelling the origin and the molecular basis of *M. tuberculosis'* evolutionary success, and lead to reconsideration of the impact of tuberculosis on human natural selection.

In the eighteenth and early nineteenth centuries, TB prevalence peaked in Western Europe and the United States and was undeniably the largest cause of death. During this time in Europe, TB was referred to as the 'White Plague' due to the colour of the skin in patients suffering from the condition (Herzog, 1998; Daniel *et al*., 1994). Until the early 1990s, there was a firm belief that tuberculosis had been conquered (at least in the economically developed world). The reality is quite different as there is more tuberculosis today than at any other time in human history (Das and Horton, 2010).

### *1.1.2. Tuberculosis epidemiology- the global burden of TB*

In 2009, there were an estimated 9.4 million incident cases (the number of new cases arising during the year) of TB globally (equivalent to 137 cases per 100 000 population) (Fig. 1.1.). This is an increase from the 9.3 million TB cases estimated to have occurred in 2007, as slow reductions in incidence rates per capita continue to be outweighed by increases in population (WHO, 2010).

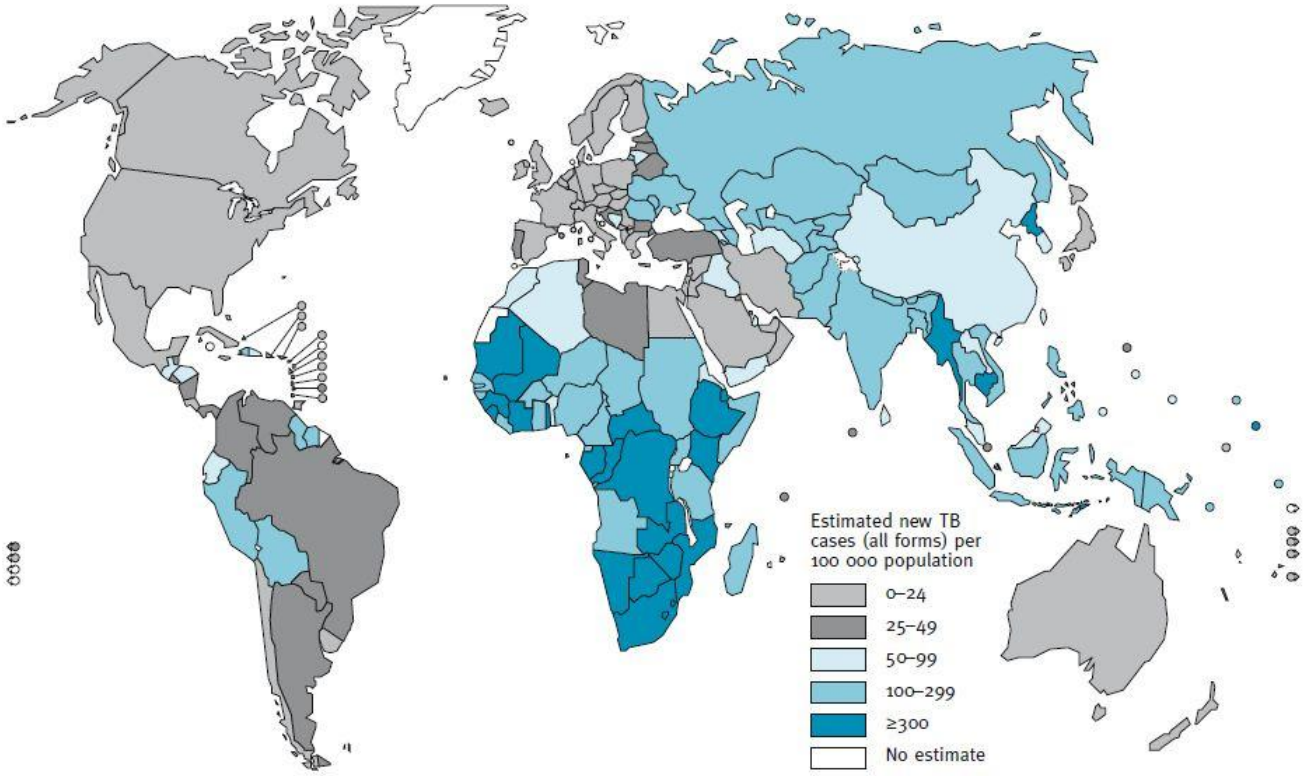


**Fig. 1.1. Estimated new TB cases (all forms) per 100 000 population, 2009 (WHO report, 2010)**

Most of the estimated number of cases in 2009 occurred in Asia (55%) and Africa (30%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (4%) and the Region of the Americas (3%).

The five countries with the largest number of incident cases in 2009 were India (1.6-2.4 million), China (1.1-1.5 million), South Africa (0.40–0.59 million), Nigeria (0.37-0.55 million) and Indonesia (0.35-0.52 million). China and India combined account for an estimated 35% of incident TB cases worldwide. Of the 9.4 million incident cases in 2009, an estimated 1.1 million (12%) were HIV-positive. Of these HIV-positive TB cases, approximately 80% were in the African Region and 13% were in the South-East Asia Region. In 2009, approximately 1.7 million people died of TB of which an estimated 0.4 million deaths were among those who were HIV-positive. The number of TB deaths per 100 000 population among HIV-negative people plus the estimated TB deaths among HIV-positive people equates to a best estimate of 26 deaths per 100 000 population (WHO, 2010).

The four countries that had the largest number of estimated cases of multidrug-resistant TB (MDR-TB) in absolute terms in 2008 were China (100 000), India (99 000), the Russian Federation (38 000) and South Africa (13 000). By July 2010, 58 countries and territories had reported at least one case of extensively drug-resistant TB (XDR-TB; WHO, 2010).

### *1.1.3. Mycobacteria*

The members of the MTC (mentioned in 1.1.1.) or 'tubercle bacilli,' are the aetiological agents of human tuberculosis (Woods, 2006). By attacking mitochondrial membranes, these organisms cause functional damage to membrane associated respiration and oxidative phosphorylation (National Jewish Medical and Research Centre, 1994). Bacteria of the genus *Mycobacterium* are aerobic, non-motile single cell non-sporulated rods. They are grouped in the suprageneric rank of actinomycetes that, unusually, have a high content (61-71 %) of guanine and cytosine (G+C) in the genomic deoxyribonucleic acid (DNA). The most prominent feature of mycobacteria that is uniformly present and distinctive to the genus is the complex, lipid-rich cell envelope. *Mycobacterium* and other closely related genera (i.e. *Corynebacterium, Gordona, Tsukamurella, Nocardia, Rhodococcus* and *Dietzia*) have similar cell wall compounds and structure, and hence show some phenotypic resemblance.

All mycobacteria consist of the same thick, hydrophobic, waxy cell wall rich in mycolic acids (α-branched lipids) which make up 50% of the dry weight of the mycobacterium cell envelope and are very strong hydrophobic molecules that form a lipid shell around the organism (Goren, 1990). These lipids may act as carbon and energy reserves. They are also involved in the structure and function of membranes and membranous organelles within the cell. The waxy coat confers the idiosyncratic characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, and distinctive immunological properties. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Barrera, 2007). The other major components of the cell wall structure of the bacteria consist of peptidoglycan and lipids. The structure of the mycobacterial cell envelope includes a plasma membrane, a peptidoglycan layer, an arabinogalactan layer esterified to an uneven mycolate layer, and a glycolipid layer. Lipoarabinomannan and a small number of porins traverse the width of the mycobacterial envelope (Woods, 2006).

Even though mycobacteria are classified with the Gram-positive bacteria, most mycobacteria do not stain well with Gram's stain. It is the complex cell envelope of mycobacteria that confers upon these bacteria the property of 'acid-fastness' (i.e. resistance to decolourisation when stained with the Ziehl-Neelsen (ZN) stain carbol-fuchsin, and decolourised with dilute hydrochloric acid or alcohol). When the bacilli are stained with this stain, they appear as red rods (Fig. 1.2.d.) and often have a beaded appearance due to their polyphosphate content and unstained vacuoles (Joklik *et al*., 1968). The smear is usually counterstained with either methylene blue or Malachite green (Fadda and Roe, 1984). Mycobacteria can be divided into two groups: fast growers and slow growers. Rapid growers are separated from slowly growing mycobacteria according to the time required to produce clearly visible colonies (≤7 days for rapid growers) (Inderlied, 2004). Most

pathogenic mycobacteria are slow growing organisms. Where non-pathogenic *M. smegmatis* has a generation time of 2-4 hours, *M. tuberculosis* has a generation time of 16-18 hours (Wayne, 1994).

#### 1.1.3.1. *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* (Fig. 1.2.) was described in March 1882 by Robert Koch, who in 1905 received the Nobel Prize in physiology or medicine for this discovery (Newton *et al*., 2000b). *Mycobacterium tuberculosis* belongs to the family Mycobacteriaceae and the genus *Mycobacterium*. This organism is also known as Koch's bacillus.

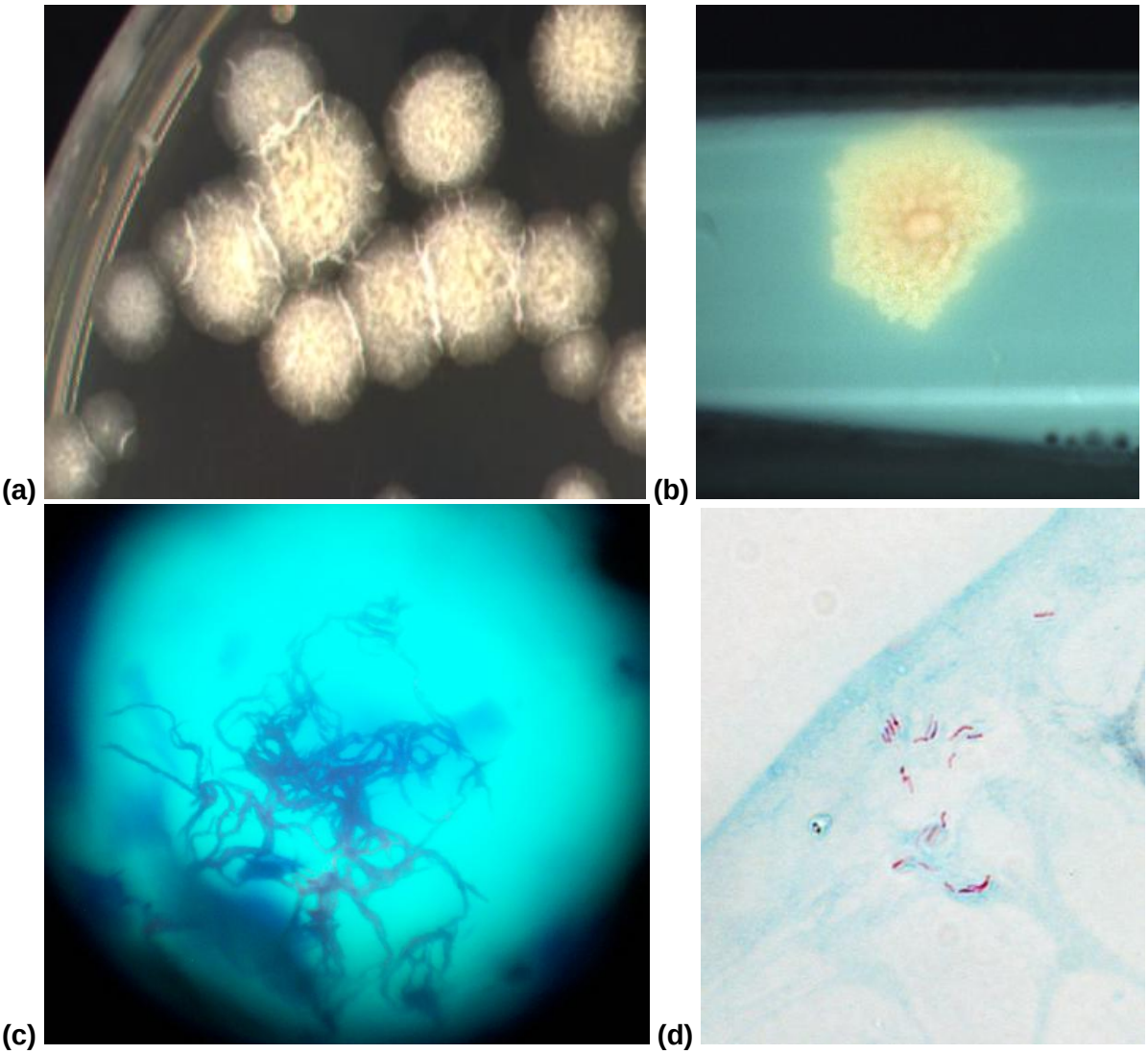


**Fig. 1.2. *Mycobacterium tuberculosis*: (a) Cauliflower appearance of *M. tb* on 7H11 agar and (b) LJ medium (c) Serpentine cording of the drug susceptible strain H37Rv under light microscope after Ziehl-Neelsen staining and (d) Ziehl-Neelsen staining of *M. tb* 1000x magnification (all photos authors own).**

Microscopically, *M. tb* is a reasonably large, slender, non-motile, slightly curved or straight, rod-shaped bacterium (Fig. 1.2.d.). The rods are 1.0 to 4.0 µm in length, 0.2 to 0.5 µm in width and the multilayered cell wall is approximately 20nm thick. Morphologically, *M. tb* cultures show typical

cream-coloured, buff and rough colonies known as the 'cauliflower appearance' when grown on solid agar or media such as the egg based Löwenstein-Jensen (LJ) medium (Fig. 1.2.a. and b.).

The serpentine cord formation of the organisms (Fig. 1.2.c.) is used as a presumptive identification method for MTC. Unlike most strains of mycobacteria other than tuberculosis (MOTT), MTC strains have the ability to form serpentine cords when grown in liquid culture media (Köksalan *et al*., 2002). This 'cording' is due to cord factor (trehalose 6,6'-dimycolate), a glycolipid consisting of two mycolic acid molecules loosely bound in the outer layer of the cell wall (Noll, 1956). A multitude of biological activities related to pathogenicity, toxicity, and protection against the host response have been attributed to this molecule. The role of the cord factor in the pathogenesis of tuberculosis is still under investigation, however, it is thought to be important because it inhibits secretion of tumour necrosis factor alpha (TNF-α) by macrophages (Brennan, 1998). However, it does not seem to be essential for bacterial multiplication *in vitro* (Indrigo *et al*., 2002).

*Mycobacterium tuberculosis* is a slow-growing bacillus which is transmitted primarily by the respiratory route. A key characteristic of *M. tuberculosis* infection is that the bacterium multiplies intracellularly, primarily in macrophages and in this way evading many host defence mechanisms (Velasco-Velazquez *et al*., 2003). Appreciating the interplay between the pathogen and the localized tissue response is critical to understanding the progression of infection to active disease, and ultimately, transmission.

### *1.1.4. The Life cycle of M. tb.: Pathogenesis of tuberculosis*

The pathogen *Mycobacterium tuberculosis* has evolved to cause infection in many, but active disease in few. The majority of individuals (90 - 95%) infected with *M. tb*, the main cause of TB, will never develop any clinical illness. Several underlying medical conditions are associated with an increased risk of progressing to tuberculosis disease (e.g., HIV infection, diabetes mellitus, renal failure, malnutrition, or advanced malignancy), but tuberculosis can develop in persons who do not have these risk factors, most probably due to genetic susceptibility (Antas *et al*., 2006; Young, 1993).

Viable *M. tb* bacilli can be present in the atmosphere as droplets or nuclei which are discharged by a person with active infection. These droplets can persist in the atmosphere for several hours and, because the infectious dose is in the range of one to ten bacilli, this makes transmission an extremely efficient process (Bloom, 1994).

Infection with *M. tb* is initiated after these inhaled bacilli are phagocytosed by alveolar macrophages, as illustrated in the life cycle diagram (Fig. 1.3.). These cells are required to phagocytose toxic and inflammatory particles and are thought to be relatively quiescent to

minimize potential damage to the lung tissue via vigorous proinflammatory responses. Once inside the phagocyte, *M. tb* modulates the behaviour of its phagosome by preventing its fusion with acidic, hydrolytically-active lysosomes, evading defence by the host (Armstrong and Hart, 1971; Armstrong and Hart, 1975; Sturgill-Koszycki *et al*., 1994; Rohde *et al*., 2007). This marks the start of a period of 'rapid' division, where the bacteria grow exponentially until the emergence of an acquired immune response.

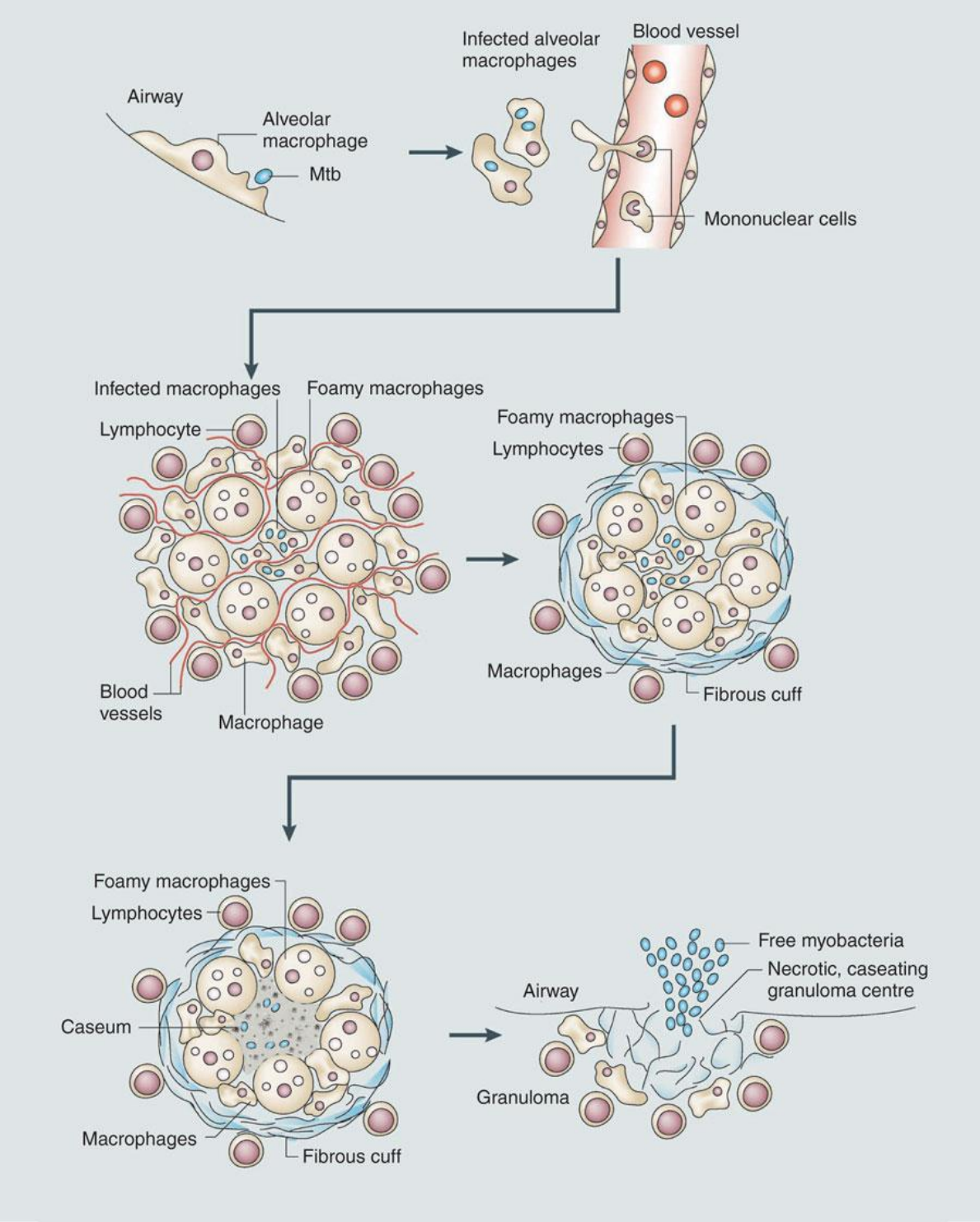


**Fig. 1.3. Life cycle of *Mycobacterium tuberculosis* (www.nature.com)**

Internalization of the bacilli triggers a proinflammatory response that induces the macrophage to invade the subtending epithelium. This response also leads to the recruitment of mononuclear cells from neighboring blood vessels. These monocytes form the cellular matrix of the early granuloma, which is the primary characteristic of tuberculosis. In its early stage, the granuloma has a core of infected macrophages enclosed by foamy macrophages and other mononuclear phagocytes, surrounded by lymphocytes.

Development of the granuloma signifies immune-mediated containment of the infection which is also the end of the period of rapid replication of *M. tb*. As the granuloma matures, it develops an extensive fibrous capsule that encases the macrophage core and excludes the majority of lymphocytes from the center of the structure. Together with this transition, there is a considerable decrease in the number of blood vessels penetrating the granuloma. At this stage there is a noticeable increase in the number of foamy macrophages in the fibrous capsule which are responsible for the accumulation of caseous debris in the center of the granuloma, which lends to the progression of active disease. In an immunocompetent individual, this progression of the granuloma can proceed to localized sterilization of the infection and mineralization of the lesion. In a progressive infection (usually in an immune compromised individual), the caseous, necrotic center of the granuloma liquefies and cavitates, spilling thousands of infectious *M. tb* into the airways. This damage to the lungs triggers the development of a productive cough, which facilitates generation of the infectious aerosol and completion of the mycobacterium's life cycle.

The primary focus of infection is usually the middle or lower zones of the lung. The bacilli are readily taken up by lung macrophages but can survive and grow to form the primary focus of infection and from there, enter the local lymphatic system and then move throughout the body via the blood and lymphatic system. This stage (the local lymphatic system), of disease is usually clinically silent or associated with mild fever and in most cases immunity develops within a few weeks and the patient becomes tuberculin positive (Girling, 1989).

Tuberculosis in the most common form is a chronic pulmonary disease classified according to three phases; primary, post-primary or miliary. Systemic features that may occur include: a persistent cough, night sweats, lassitude, weight loss, malaise, fever and anorexia (Inderlied, 2004).

The first step of possible infection is the contact between the inspired bacteria and lung alveolar epithelial mucosal cells and attending macrophages. The inhaled bacilli are phagocytosed, processed, and presented by alveolar macrophages to the T-lymphocytes. The probability of an infection depends on the ability of the mycobacterium to survive within the macrophages, in this way evading many host defence mechanisms (Fig. 1.4.). Unless a patient receives prophylaxis,

symptomatic disease eventually occurs in 5-10% of infected patients. Once the bacteria enter the macrophage, they generally locate themselves in the mycobacterial phagosome (Armstrong and Hart, 1971; Armstrong and Hart, 1975). This food vacuole derives from the plasma membrane and presents some cell surface receptors (Russell *et al*., 1996; Hasan *et al*., 1997). In contrast to normal phagocytosis, during which the phagosomal content is degraded upon fusion with lysosomes, the mycobacteria block this process (Armstrong and Hart, 1971; Armstrong and Hart, 1975). The maturation of mycobacterial phagosomes is arrested and therefore these cells are unable to eliminate bacilli by fusion of phagosomes with lysosomes. The inhibition of phagosome maturation by mycobacteria may be reverted by cytokines, such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), which also stimulate microbicidal mechanisms, including the production of reactive oxygen and nitrogen intermediates (Flesch and Kaufmann, 1991; Chan *et al*., 1992).

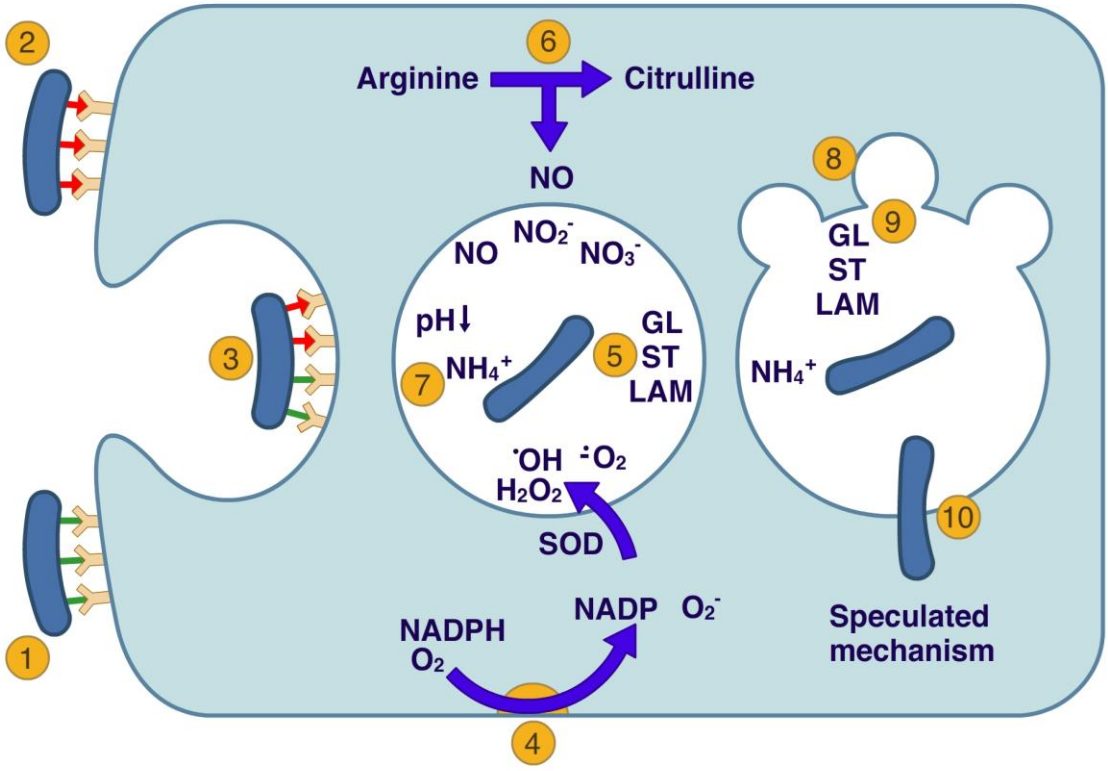


**Fig. 1.4. Macrophage phagocytosis and evasion of tubercle bacilli** *(1) The tubercle bacilli bind via lipoarabinomannan (LAM) or (2) complement receptors, (3) phagocytosis occurs with (4) the activation of an oxidative burst with superoxide dismutase (SOD), (5) glycolipids (GL), sulfatides (ST) thiols and LAM can down regulate the oxidative burst, (6) reactive nitrogen intermediates may play a role in antimycobacterial activity, as does (7) the acidic pH of the phagolysosome. Finally, (8) the production of ammonia by tubercle bacilli may diminish the effect of reactive nitrogen intermediates and contribute to (9) the failure to form a phagolysosome fusion. (10) Tubercle bacilli may evade the antimycobacterial activities of the phagolysosome by producing a hemolysin that releases the bacilli into the cytoplasm* (Inderlied, 2004).

The protective role of nitrogen intermediates has been demonstrated in different murine models (MacMicking *et al*., 1997: Flynn *et al*., 1998), and a similar function has been suggested for these molecules in human TB (Nicholson *et al*., 1996). In contrast, the role played by the reactive oxygen intermediates during infection has not been completely explained, though it is known that hydrogen peroxide produced by macrophages activated by cytokines has a mycobactericidal activity (Walker and Lowrie, 1981). Also, it has been found that the tubercle bacillus produce molecules, such as LAM, phenolic glycolipid I and thiols such as mycothiol (MSH) which work as oxygen radical scavenger molecules (Chan *et al*., 1991; Stewart *et al*., 2008).

Hematogenous spread of tubercle bacilli from the lung seldom occurs, nevertheless, hematogenous dissemination accounts for the occurrence of extrapulmonary involvement of lymph nodes, kidneys, reproductive organs, bones and gastrointestinal tract. Local features vary with respect to the organ involved.

### *1.1.5. Immunology of tuberculosis*

Initial stages of granuloma formation are dependent on TNF production by the infected macrophages and T-cells. Sustained TNF signalling is required to maintain chemokine concentrations for cellular recruitment and retention (Algood *et al*., 2005; Kindler *et al*., 1989; Roach *et al*., 2002; Saunders and Britton, 2007). Although TNF is required to drive granuloma development, too much of it can lead to overt tissue damage.

In addition to the innate virulence of the tubercle bacillus itself, the host response to *M. tb* plays a major role in determining the clinical manifestations and ultimate outcome of persons who encounter this pathogen. As previously mentioned, the majority of individuals infected with the bacillus will never develop any clinical illness although some will develop active disease in the context of some deficiency of their immune system such as that caused by infection with HIV (Young, 1993). A substantial amount of clinical experience indicates that host immunity plays an important role in the host-pathogen interaction occurring in persons exposed to *M. tb* (Schluger and Rom, 1998). Understanding the components of this host response at a basic level is likely to lead to a better understanding of the pathogenesis of tuberculosis in humans and to result in better and novel approaches to prevention and therapy of this disease.

*Mycobacterium tuberculosis* is the most conspicuous example of an intracellular bacterium that persists for long periods within the host, causing a latent infection, namely a chronic asymptomatic infection without tissue damage. This is best illustrated by the fact that two billion people worldwide are infected with *M. tb*, but more than 90% of them remain healthy and free of clinical disease and the tubercle bacilli remain within them in a state of dormancy. Therefore, although the host cell-mediated immunity is enough to control the progression of disease, it fails to exert sterile

eradication and hence, those two billion infected persons suffer the latent form of TB (Collins and Kaufmann, 2002).

The main route of entry of TB is through the respiratory tract. Alveolar macrophages and dendritic cells are the two most important cell types which mediate the fight against this pathogen (Alamelu 2004, Hickman *et al*., 2002). The initial step in the infection process of TB is inhaling the bacteria which is readily phagocytosed, processed and presented by alveolar macrophages to the T-lymphocytes (Hockings and Golde, 1979). *Mycobacterium tuberculosis* is an obligatory aerobic, intracellular pathogen that resides predominantly within macrophages. Macrophages are leukocytes cells within the tissues that originate from specific white blood cells. Macrophages develop from bone marrow precursors which mature and enter the bloodstream as monocytes. When a monocyte enters a damaged tissue through a blood vessel, it undergoes a series of changes to differentiate into macrophage. The main role of the macrophage is the removal of necrotic debris (unusual death of cells and living tissues) and dust in the lungs. The removal of dust and necrotic tissue is to a greater extent handled by fixed macrophages, which stay in strategic locations such as the lungs, liver, neural tissue, bone, spleen and connective tissue, ingesting foreign materials such as dust and pathogens, calling upon wandering macrophages if needed. When a macrophage ingests a pathogen, it will present an antigen of the pathogen to a corresponding helper T cell. Therefore macrophages can be seen as important antigen presenting cells (APC's). The antigen presentation results in the production of antibodies that attach to the antigens of the pathogens, making it easier for the macrophages to adhere to their cell membrane and phagocytose. The pathogen becomes trapped in the phagosome, which then fuses with a lysosome. Within the lysosome, enzymes and toxic peroxides digest the invader. However, some bacteria such as *M*. *tb* have become resistant to these methods of digestion (Fenton, 1998).

The entry of *M*. *tb* into the host macrophage is the key component of TB pathogenesis. Phagocytosis of *M*. *tb* by alveolar macrophages is the first event in the host-pathogen relationship that decides outcome of infection. Within 2 to 6 week of infection, cell-mediated immunity (CMI) develops, and there is an influx of lymphocytes and activated macrophages into the lesion resulting in granuloma formation. The exponential growth of the bacilli is checked and dead macrophages form a caseum. The bacilli are contained in the caseous centers of the granuloma. The bacilli may remain forever within the granuloma, or become re-activated later and may be discharged into the airways after enormous increases in numbers causing necrosis of bronchi and cavitation. When all other mechanisms have failed, fibrosis represents the last-ditch defense mechanism of the host to wall off the infection by surrounding the central area of necrosis (Alamelu, 2004). In order for *M*. *tb* to bind to monocytes macrophages, the complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR) and other cell surface receptor molecules play an important role in the binding of the organisms to the phagocytes (Schlesinger, 1994). The interaction between MR on

phagocytic cells and mycobacteria seems to be mediated through the mycobacterial surface glycoprotein lipoarabinomannan (LAM). Prostaglandin $E_2$ ($PGE_2$) and interleukin (IL)-4 (a Th2-type cytokine), regulate CR and MR receptor expression, and the interferon-gamma (IFN-γ) decreases the receptor expression, resulting in diminished ability of the mycobacteria to adhere to macrophages (Barnes *et al*., 1994). Surfactant protein receptors, CD14 receptor and the scavenger receptors also have a role in mediating bacterial binding (Gaynor *et al*., 1995). Novel approaches to therapy and new drugs are urgently needed in order to act within the host macrophages and to have direct access to the dormant organisms that presumably are within the macrophages (Quenelle *et al*., 2001).

#### 1.1.5.1. Acquired immunity

The immune system uses endogenous molecules in controlling invaders. Its most important characteristic is that these molecules can distinguish 'self' from 'non-self' and mount defense reactions in a very selective and specific manner. The immune system comprises two systems: the innate immune system and the adaptive immune system. The innate system is the first line of defense, and it is the first to clear non-self antigens (e.g. bacteria and viruses) from the body. Unlike the innate immune system, the adaptive immune system is characterized by specificity (requires the specific recognition of foreign antigens) and memory. The immunological identity of an organism and its cells are defined by surface molecules (histocompatibility complex antigens). These molecules are also involved in the modulation of immune responses (Holland and Vizi, 2002). The innate immune system has a profound influence on the type of acquired immune mechanisms generated, and *vice versa*, the specific immune response executes several of its effector functions via the activation of components of the innate immunity. Specific immune responses can be divided into cell-mediated mechanisms, which include T-cell activation and effector mechanisms, and the humoral immune response, consisting of B-cell maturation and antibody production. Both mechanisms are not mutually exclusive, and T helper cells are required for antibody maturation, isotype switching and memory B cells also function as antigen presenting cells by activating T cells in a specifically driven manner (Collins and Kaufmann, 2002).

##### *1.1.5.1.1. Cellular immune response*

Since the tubercle bacilli reside inside a compartment within the macrophage, their antigens are presented by major histocompatibility complex (MHC) class II molecules to CD4+ T lymphocytes. These cells play an important role in the protective response against *M. tb* and, when they are absent, growth of the bacilli cannot be controlled (Caruso *et al*., 1999 and Muller *et al*., 1987). This is the case in patients with an immunodeficiency, such as that caused by HIV infection. The main function of CD4+ T cells is the production of cytokines including IFN-γ, which activates macrophages and promotes bacilli destruction. Recently, another function has been ascribed to these cells, i.e., helping to develop the CD8+ T cell mediated response (Scanga *et al*., 2000;

Serbina *et al*., 2001). In the same way, CD4+ T cells may participate in the induction of apoptosis of infected cells and the subsequent reduction of bacterial viability through the CD95 Fas ligand system (Oddo *et al*., 1998). The participation of CD8+ T cells in the control of the infection is well recognized. Mice deficient in molecules such as CD8α, transporter associated with antigen processing (TAP), and perforin, were shown to be more susceptible to *M. tb* infection than animals which produced these molecules (Flynn *et al*., 1992; Behar *et al*., 1999). The mechanisms used by these cells for the control of TB seem to be mainly cytokine production and bacterial lysis. In the lungs of infected mice, CD8+ T cells showed to be able to secrete IFN-γ through activation of the T-cell receptor or by interaction with infected dendritic cells (Serbina and Flynn, 1999). Once again, the function performed by this IFN-γ is the activation of the macrophage and promotion of bacterial destruction. In addition, CD8+ T cells proved to be efficient in lysing infected cells and in reducing the number of intracellular bacteria (Stenger *et al*., 1997). The mechanisms of control of the bacterial load seem to be associated with granular exocytosis involving perforin and granzymes. Still, granulysin, which is found in CD8+ T granules, is the molecule responsible for killing the bacterium (Stenger *et al*., 1998).

#### *1.1.5.1.2. Humoral immune response*

Because of their intracellular location, it is frequently assumed that tubercle bacilli are not exposed to antibody and therefore this type of immune response is considered to be non-protective. However, during the initial steps of infection, antibodies alone or in conjunction with the proper cytokines may provide important functions, such as prevention of entry of bacteria at mucosal surfaces. As for their use in protection against TB, antibodies could enhance immunity through many mechanisms including neutralisation of toxins, opsonisation, complement activation, promotion of cytokine release, antibody-dependent cytotoxicity and enhanced antigen presentation. In this sense, data from several laboratories indicate that antimycobacterial antibodies play an important role in various stages of the host response to TB infection (Costello *et al*., 1992; Hoft *et al*., 2002; Williams *et al*., 2004; de Valliére *et al*., 2005). In particular, de Vallière *et al*. (2005) showed that specific antibodies increased the internalisation and killing of BCG by neutrophils and monocytes/macrophages. Moreover, antibody-coated BCG bacilli were more effectively processed and presented by dendritic cells for stimulation of CD4+ and CD8+ T-cell responses. The enhanced anti-mycobacterial activity of phagocytes by antibody-coated bacilli is extremely important in the context of mucosal immunity. IgG and IgA antibody classes have been shown to be present in the mucosal secretions of the human lower respiratory tract (Boyton and Openshaw, 2002). The specific mycobacterial targets for antibody-mediated enhanced interiorisation and/or killing are not known, but surface antigens such as LAM may be relevant. Taken all together, these findings suggest an urgent need to reassess the role of antibody responses in TB. In particular, the mechanism involved in antibody-mediated enhancement of

innate and cell-mediated immunity should be addressed, in order to analyze whether these mechanisms could be exploited to design alternative phytotherapeutic tools against TB.

## 1.2. Current therapy of Tuberculosis

The drugs that have been used to fight TB include isoniazid (INH), rifampicin (RIF), pyrazinamide (PA), ethambutol (EMB), streptomycin (STR), p-aminosalicylic acid (PAS), ethionamide (ETH), cycloserine, rifabutin, aminoglycosides, ciprofloxacin and ofloxacin, amithiozone, capreomycin, kanamycin and thioacetazone. Antituberculosis treatment has two main objectives (Onyebujoh *et al*., 2005). First, there is a need to rapidly kill those bacilli living extracellularly in lung cavities, which are metabolically active and are dividing continuously; this is required in order to attain the negativization of sputum and therefore to prevent further transmission of the disease. Second, it is necessary to achieve complete sterilization and elimination of those bacilli replicating less actively in acidic and anoxic closed lesions, and to kill semi-dormant bacilli living intracellularly in other host tissues, otherwise these bacilli may persist and will be responsible for subsequent TB relapses. INH is the drug with the highest activity against rapidly dividing bacilli, whereas RIF and PA have the greatest sterilizing activity against bacteria that are not dividing. These reasons, along with the prevention of drug resistance, support the use of a combination therapy for the treatment of TB.

The important first-line anti-TB drugs are INH, RIF, EMB, and PA (Bisht *et al*., 2006). Second-line drugs include the aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ETH and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin. In the near future, it is quite likely that drugs such as the rifamycin derivatives rifapentine and rifabutin and some fluoroquinolones could be incorporated into the standard antituberculosis treatment, thus being considered as first-line drugs (da Silva and Aínsa, 2007). The current short-course treatment for the complete elimination of active and dormant bacilli involves two phases:

• **initial phase**: three or more drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin) are used for two months, and allow a rapid killing of actively dividing bacteria, resulting in the negativization of sputum
• **continuation phase**: fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any remaining or dormant bacilli and preventing recurrence

Protection with bacillus Calmette-Guerin (BCG), the only vaccine available, has been disappointing, as it has shown a wide range of protection from 0 to 80% in trials carried out around the world (Roche *et al*., 1995).

## 1.3. Problem statement

Tuberculosis is a major cause of illness and death worldwide, especially in Asia and Africa. Globally, 9.4 million new cases and 1.7 million deaths from TB occurred in 2009, of which 1.1 million cases and 0.4 million deaths were in HIV-positive people. India, China, South Africa, Nigeria and Indonesia rank first to fifth respectively in terms of absolute numbers of TB cases (WHO, 2010).

Both the highest number of deaths and the highest mortality rates are in the African region. The two essential factors for the rapid spread of TB are crowded living conditions, which favour airborne transmission and a population with little natural resistance (due to HIV/AIDS). South Africa has one of the highest tuberculosis incidence rates in the world. Nearly 21 million people are infected with TB of which 10% would normally develop active tuberculosis. Not to mention that South Africa has the second highest count of HIV infections (WHO, 2010). This has severe implication due to the fact that TB is the most frequent opportunistic infection of HIV/AIDS, doubling or even tripling the clinical infection of the tubercle bacillus (Smith and Moss, 1994).

For the last 40 years there has been little progress in the treatment of Tuberculosis (Tomioka and Namba, 2006). The cocktail of RIF, INH, PA and EMB are used as the recommended first line drugs. Second line drugs, in case of organism resistance or heavy side effects, include aminoglycosides, flouroquinolones or streptomycin (CDC, 2003; Warner and Mizrahi, 2006). The long duration (6-9 months) of treatment and the lowered quality of life that goes with the adverse effects of the chemotherapy leads to patients failing to comply or adhere to the strict regime. This process leads to the increased emergence of multiple and extensively drug resistant strains (MDR-TB and XDR-TB, respectively). XDR-TB is not only resistant to both the first line drugs rifampicin and isoniazid but also to one of the second-line drugs i.e., fluoroquinolones. In addition, HIV/AIDS increases the risk for developing active TB and renders TB difficult to diagnose and treat. The TB-HIV/AIDS co-infection rate in South Africa is distressingly high, with an estimated 73 percent of new TB patients co-infected with HIV (USAID, 2009). The search for new TB treatments is a major caveat that needs to be overcome. Without the introduction of new treatments, people's options for life-saving drugs will run out.

## 1.4. Rationale

In the past, novel therapeutic approaches were often derived from natural products. Plants and plant products are present in 14 of the 15 therapeutic categories of pharmaceutical preparations recommended to medical practitioners in the western world (Phillipson and Anderson, 1989). The search for drugs and dietary supplements derived from plants has accelerated in recent years.

WHO estimates that 80% of the world's population presently uses herbal medicine for some aspect of primary health care (WHO, 2003).

Plant products have received considerable attention as potential anti-TB agents (Newton *et al*., 2000). Examples of plants that are being used to treat tuberculosis are: *Cryptocarya latifolia*, *Chenopodium ambrosioides*, *Euclea natalensis*, *Ekebergia capensis*, *Helichrysum melanacme*, *Nidorella anomala*, *Polygala myrtifolia* and *Thymus vulgaris* (Lall and Meyer, 1999). Subjecting three traditionally used South African plants (*Knowltonia vesicatoria* syn. *Anemone vesicatoria*, *Pelargonium sidoides* and *Euclea natalensis*) to *in vitro* assays can help towards finding new therapeutic treatments for the depleting immune system and the virulence of *M. tb* in TB-sufferers.

Although most traditionally used plant therapies rely for their effects on a variety of compounds and synergy between these compounds, there are numerous benefits for isolating and identifying active constituents from these bioactive plants. Characterising toxicity profiles, simpler determination of modes of action and new activities of known compounds adds to the wealth of information on phytochemicals. Combining plant extracts and current TB drugs holds advantages such as decreased toxicity profiles, increased activity and reduced onset of microbial resistance. Testing isolated compounds from plant extracts, using whole extracts alone, in combination with other extracts and current anti-TB drugs covers a wider range for activity and possible treatment therapies.

Testing extracts and compounds with antituberculous activity for the possible mechanism of action can only be beneficial to promote novel drug development. A Glutathione reductase enzyme assay can be employed as a surrogate for the *M. tb* enzyme (Mycothione reductase, Mtr) to screen samples for selective enzyme inhibition as a possible mechanism of action. Mycothione reductase maintains a reduced state within *M. tb* organisms by reducing mycothione (MSSM) to mycothiol (MSH) which is the main free radical scavenging molecule present in Mycobacteria. Naphthoquinones (isolated from *Euclea natalensis*) are plant compounds that have recently shown considerable potential as anti-TB agents, especially considering their thwarting activity on the Mtr/MSSM/MSH redox-pathway (McGaw *et al*., 2008; Mahapatra *et al*., 2007).

*Mycobacterium tuberculosis* is primarily an intracellular pathogen residing in the acidic vacuoles of macrophage cells. This environment may affect the action of anti-TB drugs such as streptomycin (activity reduced) and pyrazinamide (activity increased; Newton *et al*., 2000b) and it may be valuable to evaluate the ability of plant compounds to inhibit *M. tb* within cultured human macrophages. In addition, understanding the components of the host response at a basic level is likely to lead to a better understanding of the pathogenesis of tuberculosis in humans and to result in better and novel approaches to protection and therapy of this disease. Infecting cultured

macrophages with *M. tuberculosis* and measuring cellular cytokine levels in these cells during infection and during treatment with plant extracts, could help in developing and testing hypotheses about host immunity during tuberculosis and the related plant extracts effect on cytokine levels and hence immune responses.

## 1.5. Hypotheses

Tannins do not account for the antimycobacterial activity of the aerial ethanol extract of *Knowltonia vesicatoria*.

Synergy of compounds within the aerial ethanol extract of *Knowltonia vesicatoria* accounts for its antimycobacterial activity.

Biological activity of the aerial ethanol extract of *Knowltonia vesicatoria* will increase with different combinations of the *Euclea natalensis* chloroform and *Pelargonium sidoides* ethanol root extracts and the antitubercular drug Isoniazid.

## 1.6. Aims and objectives

1. Investigate the antimycobacterial activity of the tannin-free *K. vesicatoria* compared to the crude EtOH extract against *M. tb*.
2. Determine the possible synergistic antimycobacterial activity of three combinations against *M. tb*:
    i) Combination 1 (C1): the most active extract of *K. vesicatoria* (tannin-free or crude EtOH) and the EtOH extract of *Pelargonium sidoides* combined with the chloroform ($CHCl_3$) extract of *Euclea natalensis*.
    ii) Combination 2 (C2): the active extract of *K. vesicatoria*, the EtOH extract of *Pelargonium sidoides* and the chloroform ($CHCl_3$) extract of *Euclea natalensis* combined with the antitubercular drug INH.
    iii) Combination 3 (C3): the active extract of *K. vesicatoria* combined with the antitubercular drug INH.
3. Isolate and identify the bioactive compound(s) from *K. vesicatoria* and determine the antimycobacterial activity against *M. tb*.
4. Investigate glutathione reductase inhibitory or subversive substrate activity of samples found to be active against *M. tb*.
5. Determine cytotoxicity of samples found to be active against *M. tb*.
6. Investigate intracellular antimycobacterial activity of samples found to be active against *M. tb*.
7. Explore the immunomodulatory properties of samples found to be active against *M. tb*.

## 1.7. Scope of dissertation

### *1.7.1. Selection, collection, extraction and antimycobacterial activity of plant material*

The problems and potential of natural products as novel medicinal therapies is discussed, with focus on plants used and/or tested for antimycobacterial activity. Three plants in three distinct families (Ebenaceae, Geraniaceae and Ranunculaceae) were selected for the current study, on the basis of ethnobotanical uses as immune boosters or for the treatment of tuberculosis, colds and flu. The antimycobacterial activity of these plant extracts alone and as three synergistic combinations (as described in section 1.6.) is investigated.

### *1.7.2. Identification and isolation of bioactive compounds from Knowltonia vesicatoria*

It was previously found that *K. vesicatoria* possessed high antimycobacterial activity. The objective was to isolate the active compound(s) and evaluate the minimal inhibitory concentration (MIC) of these compounds against *M. tuberculosis*.

### *1.7.3. Inhibitory and subversive substrate activity of selected antitmycobacterial samples towards glutathione reductase*

The possible mechanism of action of the selected antimycobacterial extracts and compounds is investigated. *Mycobacterium tuberculosis* lacks glutathione, but instead maintains millimolar concentrations of the structurally distinct low molecular weight thiol MSH. MSH has antioxidant activity as well as the ability to detoxify a variety of toxic compounds. Because of these activities, MSH is a candidate for protecting *M. tuberculosis* from inactivation by the host during infections as well as for resisting antituberculosis drugs. In order to define selective mechanism of the inhibitory and subversive substrate activity of selected antituberculosis samples, extracts, synergistic combinations and compounds were tested against glutathione disulfide reductase as a model for mycothiol disulfide reductase.

### *1.7.4. Cytotoxicity, intracellular antimycobacterial activity and immunomodulation of extracts, combinations and compounds*

Cytotoxicity of the selected plant extracts, synergistic combinations and compounds was determined using U937 cells (human macrophages) with the intention of choosing the plant extract with the lowest toxicity and the highest intracellular anti-TB activity and intracellular cytokine level measurement. Local cellular immune responses affect presentation and outcome in tuberculosis. According to Condos *et al*. (1998), with treatment and clinical improvement, patients with active pulmonary TB recruit cytokines such as IFN-γ to the lung. To investigate the hypothesis that a

plant extract, mixture or compound will increase specific cytokine levels intracellularly in single cell cultures, the level of seven cytokines (Interleukin-2,-4,-6,-10,-12, Interferon gamma and Tumour necrosis factor alpha) was measured in U937 macrophages treated with selected samples.

## 1.8. Structure of dissertation

The contents of each chapter are as follows:

**Chapter 1:** An introduction to tuberculosis, its epidemiology, pathogenesis and immunology. The rationale behind the study, the hypothesis and the layout of the dissertation.

**Chapter 2:** Literature, description and phytochemical constituent information of the plants selected for antimycobacterial, synergistic and immunomodulatory activity. The collection, extract preparation and antimycobacterial activity of the plant material.

**Chapter 3:** Isolation of compounds present in the crude ethanolic extract of *K. vesicatoria*. Antimycobacterial activity of the isolated compounds against *M. tuberculosis* strains.

**Chapter 4:** Glutathione disulfide reductase activity of the selected extracts and compounds.

**Chapter 5:** Cytotoxicity of extracts and compounds on differentiated U937 macrophages. Intracellular antimycobacterial activity of these samples. Cytokine profile of sample treated macrophages.

**Chapter 6:** General discussion and conclusion

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# CHAPTER 2
# SELECTION, COLLECTION, EXTRACTION and ANTIMYCOBACTERIAL ACTIVITY of PLANT MATERIAL

## 2.1. Introduction

### *2.1.1. Natural products: Potential and problems*

Natural sources offer a wealth of chemically diverse compounds that have been evolutionary preselected to modulate biochemical pathways. Biologically active compounds have been found among many skeleton types, mainly from plants, but also from other organisms such as fungi, bacteria and marine organisms. Medicinal plants have been used for their curative properties by traditional healers for centuries (Hutchings *et al*., 1996; van Wyk *et al*., 1997). Phytotherapy (therapeutic use of plants) is an integral part of all traditional medicinal doctrines around the world. Although these different systems (from Ayuverda and Unani to Chinese medicine) are based on different theoretical and cultural models, they all integrate medicinal plants into their dogma (WHO, 2007).

The literature documents the use of plants for a range of conditions and, as a consequence, Western scientists have become increasingly interested in the rationale behind the use of traditional remedies. Plants and their derivatives contribute to more than 50% of all medicine used worldwide (Mander, 1999). Many modern medicines have their origins in plants that are often used in the treatment of illness and disease. Salicylic acid, a precursor of aspirin, was originally derived from the bark of the White Willow (*Salix alba*) and flowers from the Meadowsweet plant (*Filipendula ulmaria*). Well known examples of plant derived drugs include the antimalarial quinine, extracted from the bark of the *Cinchona* species. Vincristine, which is being used to treat certain types of cancer, comes from the Madagascar periwinkle (*Catharanthis roseus*). In 1819, the isolation of the analgesic morphine, codeine and paregoric laid down the foundation for the purification of pharmacologically active compounds for the treatment of diarrhoea. ‘Laudanum’, a tincture of the Opium Poppy (*Papaver somniferum*), was the favoured tranquilliser in Victorian times. Even today, morphine, the most important alkaloid of the opium poppy, remains the standard against which new synthetic analgesic drugs are measured (Phillipson, 2001 and De Smet, 1997).

There is a surplus of structures that have been reported to have anti-TB activity as summarized in three recent reviews, one focusing on plant terpenoids (Cantrell *et al*., 2001), and the others

providing a comprehensive summary by structural types and including other organisms as well (Copp, 2003; Okunade *et al*., 2004). A report from Newton *et al* (2000) is also an excellent source of information of plant species with promising anti-TB activity (Table. 2.1.).

The problem is that none of these above mentioned, non-microbial natural products, with antimycobacterial activity have moved forward in drug development. Despite changing strategies in natural product research, which concern sample selection and collection, isolation techniques, structure elucidation, biological evaluation, semisynthesis, dereplication, biosynthesis, as well as optimisation of downstream processing the rate of discovery of truly novel natural product has actually decreased (Monks *et al*., 1997; Veral, 1996).

According to Pauli *et al* (2005) there are indeed exceedingly valid aspects to consider when trying to explain the complications associated with natural product research: (i) compound availability is very low (low yield, one-sample-one-source problem and related resource issues), (ii) relative structural complexity is very high and includes the occurrence of multiple stereoisomers (e.g., most triterpenes contain 10 or more chiral centres), (iii) follow up studies are mostly lacking, since most efforts (e.g. in academic environments) are not part of focused drug development programs and simply lack the opportunity for e.g. synthetic follow-up of promising leads; (iv) the isolated active principles rarely exhibit potent activity (MIC ≥1 µg/ml) themselves, but require follow up improvement in order to be attractive. High toxicity and low bioavailabilty of plant derived compounds found to be highly active *in vitro* is another important drawback that goes hand in hand with points (iii) and (iv). To add to this, the field of plant based natural drug development is interdisciplinary. Plainly put, institutions and individuals do not have access to all the knowledge and expertise that encompasses the taxonomy, chemistry and biology associated with this type of research.

No research field is without problems and challenges. The process of combining phytotherapy with the scientific medical model is aimed at demonstrating the safety and efficacy of single or combined medicines that have been used in the past millennia. Even with the rich plethora of structures and plants that have been researched for antimycobacterial activity, only a small proportion of all the plant species have been thoroughly investigated and undoubtedly there are many novel biologically active compounds and therapeutic combinations still to be discovered.

**Table. 2.1. Various plants with antimycobacterial activity (selected from Newton *et al*., 2000)**

| Plant Species (Family) | Origin | Plant Part | Mycobacteria species used | Active Constituent(s)/ extract(s) | Activity | Side effects/ toxicity | Remarks |
|---|---|---|---|---|---|---|---|
| *Actaea spicata* (Ranunculaceae) | - | - | *M. tb* | Ethanol extract | Inhibitory dilution (no growth or less than 5 colonies) = 1:320 | - | High activity |
| *Adhatoda vasica* (Acanthaceae) | India | Leaf | BCG<br>*M. tb* | Bromhexine and Ambroxol (semi-synthetic derivatives of alkaloid vasicine) | Average MIC for 5 clinical isolates of *M. tb* with ambroxol was 64 µg/mL<br>Average MIC for bromhexine was 128 µg/mL for 3 clinical isolates of *M. tb* | - | Ambroxol well tolerated clinically administered orally up to 500 mg twice daily |
| *Borrichia frutescens* (Asteraceae) | USA | Flower<br>Leaf<br>Stem | *M. tb* | **1.** (24R)- 24,25-epoxycycloartane<br>**2.** (3aH, 24R)-24,25-epoxycycloartane<br>and<br>**3.** (23R)-3-oxolanosta-8,24- dien-23-ol<br>All isolated from Dichloromethane extract | MIC<br>**1.** 8 µg/mL<br>**2.** 8 µg/mL<br>**3.** 128 µg/mL | $IC_{50}$ (Vero cells)<br>**1.** 71.8 µg/mL<br>**2.** 39.8 µg/mL<br>**3.** 103.6 µg/mL | Highest level of anti-mycobacterial activity found in flower extract |
| Brucea javanica (Simaroubaceae) | - | - | *M. tb* | Bruceoside-D (Quassinoid) | 7% inhibition of *M. tb* at 12.5 µg/mL | - | - |
| *Cetraria islandica* (Parmeliaceae) | Iceland | - | *M. aurum* | Proto-lichesterinic acid | MIC = 250 µg/mL | - | Reputed to be effective in treatment of pulmonary tuberculosis |
| *Coptis coinensia* (Ranunculaceae) | China | Root | *M. tb* | Berberine bisulphate (alkaloid) | MIC = 1:800 dilution | Berberine highly toxic by parenteral injection | Significant activity but highly toxic |
| *Empetrum nigrum* (Empetraceae) | British Columbia | Stem | *M. tb*<br>*M. avium* | Methanol extract | Complete inhibition of both mycobacteria at 50 µg extract/disc | - | Significant activity |

**Table. 2.1. Continued: Various plants with antimycobacterial activity (selected from Newton *et al*., 2000)**

| Plant Species (Family) | Origin | Plant Part | Mycobacteria species used | Active Constituent(s)/ extract(s) | Activity | Side effects/ toxicity | Remarks |
|---|---|---|---|---|---|---|---|
| *Erigeron philadelphicus* (Asteraceae) | - | - | *M. tb*<br>*M. avium* | Matricaria lactones | MIC (µg/mL), for both organisms, ranged from 12.5 - >100 for each of the compounds tested | - | Significant activity of 4Z, 8Z-matricaria ester which gave a MIC of 12.5 µg/mL against *M. tb* |
| *Ferula communis* (Umbelliferae) | Saudi Arabia | Rhizome | *M. intracellulare*<br>*M. xenopi*<br>*M. chelonae/chelonei*<br>*M. smegmatis* | Ferulenol (Coumarino-sesquiterpene) | MIC (µg/mL) of 1.25 for each of the mycobacterial species | - | Significant activity.<br>Ferchromone was another compound identified<br>which had an MIC of 50 µg/mL for each of the<br>mycobacterial species |
| *Galipea officinalis* (also known as *Cusparia febrifuga*; Rutaceae) | - | Bark | *M. tb* | Ethanol extract<br>Alkaloids<br>**1.** Cusparine<br>**2** .Galipine<br>**3.** 4-methoxy-2-npentylquinoleine<br>**4.** N-Methyl-2-quinolone | MIC (µg/mL)<br>Ranged from 6.25-629 depending on the fraction and the strain of *M. tb* | - | - |
| *Glycyrrhiza glabra* (Leguminoseae) | China | - | *M. tb*<br>*M. smegmatis* | Licoiso-flavone (Flavonoid) | MIC against *M. smegmatis* (50 µg/mL) and *M. tuberculosis* (25 µg/mL) | - | - |
| *Humulus lupulus* (Cannabinaceae) | - | Flower | *M. tb* | Ethanol extract | Active (concentration not stated) | - | - |
| *Hypericum calycinum* (Hypericaceae) | - | Leaf | *M. tb* | Ethanol extract | Active (concentration not stated) | - | - |
| *Inula helenium* (Asteraceae) | USA | Root | *M. tb* | Dichloromethane and hexane extracts | 100% inhibition against *M. tb* at 100 µg/mL | - | - |

**Table. 2.1. Continued: Various plants with antimycobacterial activity (selected from Newton *et al*., 2000)**

| Plant Species (Family) | Origin | Plant Part | Mycobacteria species used | Active Constituent(s)/ extract(s) | Activity | Side effects/ toxicity | Remarks |
|---|---|---|---|---|---|---|---|
| *Ipomoea purga* (Convolvulaceae) | - | - | *M. tb* | Ethanol extract | MIC (no growth or less than 5 colonies) = 1:160<br>Partial inhibition = 1:320 dilution | - | Significantly active, but maybe unsuitable due to purgative property |
| *Juniperus excelsa* (Cupressaceae) | Saudia Arabia | Leaf | *M. intracellulare*<br>*M. xenopi*<br>*M. chelonae*<br>*M. smegmatis* | Ethanol extract followed by partition between n-hexane and acetonitrile Ethanol extraction yielded<br>**1.** Ferruginol<br>**2.** Sandaracopimeric acid<br>**3.** Hinokinol<br>**4.** 3b-hydroxy-sandaracopimeric acid | **1.** 5 µg/mL against each species<br>**2.** 32 µg/mL (only tested on *M. smegmatis*)<br>**3.** Inactive (only tested on *M. smegmatis*)<br>**4.** Not tested | - | - |
| *Karwinskia humboldtiana* (Rhamnaceae) | Mexico | Root | *M. smegmatis* | Dichloromethane and ethanol extract.<br>Karwinaphthol A<br>Karwinaphthol B | Karwinaphthol A inhibited growth at 12.5 µg/mL and Karwinaphthol B at 50 µg/mL | - | - |
| *Lavandula angustifolia* (Labiatae) | France | Flower | *M. chelonae/chelonei*<br>*M. fortuitum*<br>*M. kansaii*<br>*M. marinum*<br>*M. scrofulaceum* | Essential lavandino oils | Diameter of the zones of inhibition (in mm) ranged from 20-35 depending on the year the samples were harvested and the species tested against. | - | Significant antimycobacterial activity on all species |
| *Leucas volkensii* (Labiatae) | Kenya | Aerial | *M. tb* | Methanol extract<br>**1.** (E)-phytol<br>**2.** Phytanol<br>**3.** (Z)- phytol<br>**4.** Mixture of E)-and (Z)-phytol<br>**5.** Geraniol<br>**6.** Farnesol | MIC<br>**1.** 2 µg/mL<br>**2.** 2 µg/mL<br>**3.** 2 µg/mL<br>**4.** 2 µg/mL<br>**5.** 64 µg/mL<br>**6.** 8 µg/mL | - | Significant activity from all compounds.<br>(E)-phytol, phytanol, (Z)-phytol and the mixture of<br>(E)- and (Z)-phytol were the most active and their activities were in same range as ethambutol (0.95-3.8 µg/mL) |

**Table. 2.1. Continued: Various plants with antimycobacterial activity (selected from Newton *et al*., 2000)**

| Plant Species (Family) | Origin | Plant Part | Mycobacteria species used | Active Constituent(s)/ extract(s) | Activity | Side effects/ toxicity | Remarks |
|---|---|---|---|---|---|---|---|
| *Mammea americana* (Guttiferaceae) | Puerto Rico | Leaf | *M. tb*<br>*M. smegmatis* | Ethanol | 10mm inhibition zone at 25 µg/disc for *M. smegmatis*<br>Activity towards *M. tb* at 50 µg | $LC_{50}$ = 224.6 mg/mL (Brine shrimp).<br>No toxicity to mice at 500 mg/100 mL of plant extract given i.p. over 15 days | Bactericidal inhibitory pattern on MT growth comparable to that of streptomycin |
| *Melia volkensii* (Meliaceae) | Kenya | Seeds | *M. tb* | Methanol extract<br>**1.**12β hydroxykulactone<br>**2.** 6β hydroxykulactone<br>**3.** Kulonate | MIC<br>**1.** 16 µg/mL<br>**2.** 4 µg/mL<br>**3.** 16 µg/mL | Poisonous at high levels | - |
| *Nuphar lutea* (Nymphaceae) | British Columbia | Rhizome | *M. tb*<br>*M. avium* | Methanol extract | Complete inhibition of at *M. tb* 50 µg extract/disc. Small zone of clearing of *M. avium* with 50 µg extract/disc | - | - |
| *Oplopanax horridus* (Araliaceae) | North America | Inner bark | *M. tb* and Isoniazid resistant *M. avium* | **1.** Falcarindol<br>**2.** Falcarinol<br>**3.** Oplopandiol<br>**4.** Active oil 1 ($C_{20}H_{28}O_4$)<br>**5.** Active oil 2 ($C_{20}H_{30}O_4$)<br>All polyynes isolated from extraction with methanol followed by dichloromethane | All active constituents, methanol and chloroform extracts were active at a concentration of 10 µg/disc against *M. tb* and isoniazid resistant *M. avium* Falcarinol and active oil 2 ($C_{20}H_{30}O_4$) completely inhibited growth of *M. tb* and isoniazid resistant *M. avium* at 20 µg/disc | | |
| *Pieris japonica* (Ericaceae) | - | Leaf | *M. tb* | Water | MIC (complete inhibition) = 1:640 dilution | - | Significant activity |
| *Piper cubeba* (Piperaceae) | Indonesia | - | *M. tb* | Ethanol | MIC (no growth or less than 5 colonies) = 1:320 | - | High specific activity. |

**Table. 2.1. Continued: Various plants with antimycobacterial activity (selected from Newton *et al*., 2000)**

| Plant Species (Family) | Origin | Plant Part | Mycobacteria species used | Active Constituent(s)/ extract(s) | Activity | | Side effects/ toxicity | Remarks |
|---|---|---|---|---|---|---|---|---|
| *Rudbeckia submentosa* (Asteraceae) | - | Root<br>Leaf<br>Stem<br>Flower | *M. tb* | **1.** Allolantolactone 3<br>**2.** oxoalloalantolactone isolated from dichloromethane extract | 100 µg/mL dichloromethane extract of root gave 99% inhibition.<br>MIC<br>**1.** 32 µg/mL<br>**2.** 128 µg/mL | - | Compounds previously isolated from *Eupatorium quadrangularae* | |
| *Salvia multicaulis* (Labiatae) | Turkey | Root | *M. tb* | **1.** Norabietane 1<br>**2.** Norabietane 2<br>**3.** Norabietane 3<br>**4.** Norabietane 4<br>**5.** Abietane 1<br>**6.** Abietane 2<br>**7.** Primarane<br>All isolated from an acetone soluble extract | MIC<br>**1.** 5.6 µg/mL<br>**2.** 0.46 µg/mL<br>**3.** 2.0 µg/mL<br>**4.**1.2 µg/mL<br>**5.**1.2 µg/mL<br>**6.** 0.89 µg/mL<br>**7.** 7.3 µg/mL | - | Test compounds **1**-**7** gave comparable values to standard anti-tubercular agents i.e. rifampicin | |
| *Strobilanthus cusia* (Acanthaceae) | China/Taiwan | | *M. tb*<br>*M. smegmatis*<br>*M. avium* complex (MAC) | Tryptanthrin (indolequinazolinone alkaloid) | MIC<br>1 µg/mL *M. tb*<br>4 µg/mL *M. smegmatis*<br>2 µg/mL MAC | | High significant activity. Also active against drug sensitive and drug resistant *M. tb* and MAC. Potency of compound in same range as that of Antitubercular drugs already in use, i.e. streptomycin and ethambutol. Compound also isolated from *Polygonum tinctorium* and *Isatis tinctoria* | |
| *Tetradenia riparia* (Lamiaceae) | Rwanda | Leaf | *M. tb* | Diterpenediol in ethanol extract | Activity against *M. tb* ranging between 25-100 µg/mL, depending on strain | - | - | |
| *Zingiber officinale* (Zingiberaceae) | - | Rhizome | *M. tb*<br>*M. avium* | **1.** 10-gingerol<br>**2.** 8-gingerol<br>**3.** 6-gingerol isolated from dichloromethane extract | MIC (mg/mL)<br>**1.** 25 (*M. avium*)<br>50 (*M. tb*)<br>**2.** 50 (For both)<br>**3.** >100 (For both) | - | - | |

$IC_{50}$- Fifty percent inhibitory concentration; i.p- intra peritoneal; $LC_{50}$- Fifty percent lethal concentration; MIC- Minimum inhibitory concentration; *M. tb- Mycobacterium tuberculosis*

### *2.1.2. Synergism*

The general understanding of synergy is that it is an effect seen by a combination of substances being greater than would have been expected from a consideration of individual contributions. This can apply to either an increased therapeutic effect, a reduced profile of side effects or, preferably and logically, both (Williamson, 2001).

Combinations of herbs are fundamental to the philosophy of Western medical herbalism, traditional Chinese medicine and Ayurveda as well as being due to empirical observations and historical usage. There are substantial reasons for not isolating individual components found in popular phytomedicines such as St. John's Wort, Echinacea, Aloe, Ginkgo and garlic. These herbs are traditionally used as standardized extracts for which we have considerable pharmacological and clinical evidence with positive data to support their use. Synergy is generally assumed to play a part in their medicinal effects (Williamson, 2002). Synergistic interactions are of vital importance in phytomedicines, to explain difficulties in always isolating a single active ingredient, and explain the efficacy of apparently low doses of active constituents in a herbal product. This concept, that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient, also underpins the philosophy of herbal medicine. Evidence to support the occurrence of synergy within phytomedicines is now accumulating (Williamson, 2001).

Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health, treat an illness through polyvalent pharmacological effects or attack a specific pathogen via different means of inhibition. The term ‘polyvalent (synonyms are: multivalent or pleiotropic) action’ denotes an improved and co-operative sort of effect and this usually refers to the treatment of an illness as a whole (Williamson, 2001). A single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swelling and pain, phenolic compounds that can act as an antioxidant, antibacterial and antifungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give an overall sense of well-being (Gurib-Fakim, 2006).

Development of new drugs to treat TB faces even more constraints than the development of therapeutic agents for other diseases. This is due, in part, to intrinsic properties of the tubercle bacillus, such as its slow growth, phenotypic drug resistance during persistence and the need for compounds with a novel mode of action because of the increasing prevalence of primary resistance to the current TB drugs. Demographic changes to the population of TB patients are also a confounding factor; these now include co-infection with HIV, but other elements, such as the growing type-2 diabetes epidemic, should not be ignored. Consequently, a new TB drug will not only have to pass all the safety requirements associated with prolonged administration but also

have to be compatible with antiretroviral therapy and, possibly, other medications (Balganesh *et al*., 2008). Few new agents are in development today for treating TB, and none have been designed specifically to shorten the treatment regimen and provide the breakthrough in therapy that is sorely needed if the epidemic is to be brought under control. Drug design targeting the latency stage and synergistic interaction between the various drug candidates might prove to be good alternatives (Hemaiswarya *et al*., 2008).

Antimycobacterial treatment has always been a combination therapy. Today's TB treatment, which dates back to 1970s, is long and burdensome, requiring at least six months of multidrug chemotherapy. In clinical practice, a combination of various drugs is necessary to prevent the development of drug resistance during TB therapy, studies of drug combinations are therefore essential (Bergmann and Woods, 1998). Better TB treatments will still require drugs to be taken in combination, in order to reduce TB's six month treatment time, be effective against drug-resistant strains, and be compatible with anti-retroviral (used to treat patients with TB-HIV co-infection) and diabetes therapy. A true polyvalent treatment for TB would be a combination of compounds that both enhance the antimycobacterial effects and augments immune function.

Synergistic interaction between plant-derived compounds is gaining sufficient attention from researchers (Chen *et al*., 2006). One of the best examples of a plant derived compound that enhances drug activity is the alkaloid piperine (1-piperoyl piperidine) from Black and Long Pepper (*Piper nigrum* and *Piper longum*). The combination of piperine with essential drugs, such as antibiotics, antihypertensive and antiepileptics as well as nutrient supplements, led to dose economy due to enhanced uptake, higher blood concentration and the drug being available for a longer duration in the body (Atal *et al*., 1985; Hiwale *et al*., 2002; Khajuria *et al*., 1998; Lambert *et al*., 2004; Singh *et al*., 2005). Piperine enhances bioavailability of nutrients and drugs by slowing down drug metabolism and biodegradation (Majeed and Prakash, 2000). Synergy has recently been identified as a major factor in explaining the overall antimicrobial activity of plant-derived agents. An ethyl acetate fraction of the EtOH extract of *Moringa oleifera* pods increased plasma concentrations of the antituberculosis drug RIF due to the inhibition of the drug metabolizing enzyme cytochrome P-450 (Pal *et al*., 2010). Another reason for the increased bioavailability could be the enhanced blood supply in enteric vessels as a result of vasodilatation induced by niazirine isolated from *M. oleifera* (Faizi *et al*., 1994). A flavonolignan, 5'-methoxyhydnocarpin, inhibits multi-drug resistance pumps which significantly enhance the antimicrobial effect of berberine alkaloids contained in the same *Berberis* extracts (Stermitz *et al*., 2000).

Combination studies with natural products from plants and synthetic drugs are limited to a few reports. Totarol, ferulenol and plumbagin were observed to increase the potency of INH by fourfold against *Mycobacterium* sp. (Mossa *et al*., 2004). The naphthoquinone 7-methyljuglone (7-MJ),

isolated from the roots of *Euclea natalensis* in combination with isoniazid or rifampicin resulted in a four- to six-fold reduction in the MIC of the synthetic drugs (Bapela *et al*., 2006). An aqueous extract from *Cuminum cyminum* seeds produced a 35% enhancement of rifampicin levels in rat plasma. This activity was due to a flavonoid glycoside, 3',5-dihydroxyflavone-7-*O*-β-D-galacturonide 4'-*O*-β-D-glucopyranoside, found in the natural product. The altered bioavailability profile of rifampicin could be attributed to the permeation enhancing effect of this glycoside (Sachin *et al*., 2007).

Secondary metabolites from plant are good sources for combination therapy. Synergy would pave a new strategy for the treatment of TB, overcome drug-resistance, and decrease the use of antibiotics and hence the side effects created by them. There are a wide range of phytochemicals which act as multidrug resistance modifiers with their mechanism of action ranging from inhibiting or blocking resistant receptor or active sites, inhibiting enzymes that specifically degrade or modify the therapeutic drug, blocking of efflux pumps (bacterial system of exporting the drug from within the bacteria outwards) and increased membrane permeability (Hemaiswarya *et al*., 2008). There are no reports on the use of natural products and synthetic drug combinations used in the clinical settings. There is plenty of hope for natural products to be used in combination with antibiotics as anti-infective drugs.

### *2.1.3. Tannins: Unwanted polyphenolics*

Tannins are plant polyphenols biosynthesized via the Shikimic acid or acetate pathway. These secondary metabolites are widespread in plants, and although they may serve as an effective defence against herbivores, their role is largely unclear (Gurib-Fakim, 2006). The chemistry of these compounds is very complex. Two broad groups are recognised, namely hydrolysable tannins (polyesters of gallic acid and/or hexahydroxydiphenic acid and their derivatives) and condensed tannins (proanthocyanidins containing oligomers of flavandiol; Jones and Kinghorn, 2005). This distinction is based on whether acids or enzymes can hydrolyse the tannin compound components or whether they condense the components to polymers (Gurib-Fakim, 2006).

Tannins have been known to exhibit biological activity due to their intrinsic protein binding and protein inactivation. Many tannins give false-positive results in various biological assays, usually because of their tendence to form non-selective complexes with proteins (enzymes, receptors and structural proteins) through multipoint hydrogen bonding (Haslam, 1998). Organic extracts containing tannins inhibit enzymes such as topoisomerase I and II, viral transcriptase etc, leading to false positive results (Jones and Kinghorn, 2005).

It is well known that the tannins inhibit absorption from the intestine (Houghton *et al*., 2007) which may also hinder the absorption of proteins, alkaloids and therapeutic drugs (Williamson, 2001).

Due to the absorption inhibition of tannins, they are used against diarrhoea and as an antidote in poisoning by heavy metals (Gurib-Fakim, 2006). Although recent studies have reported that tannins have anti-cancer and anti-HIV activities (Gurib-Fakim, 2006), their use declined after the discovery of hepatotoxic effects of absorbed tannic acids and the induction of enzymes such as cytochrome P450 which may accelerate drug metabolism resulting in blood levels of drug actives too low for a therapeutic effect (Williamson, 2001). Tannins are therefore unwanted compounds in the search for novel anti-bacterial and antimycobacterial phytochemicals. The removal of tannins from a previously biologically active plant extract can render the extract inactive, if the extract still exhibits the same biological activity without the presence of tannins, then the activity is due to other phytochemicals.

## 2.2. Selection of plants

The first step in drug development from naturally derived products, such as plants, is selection of those species that would most probably contain the sought after biological activity. Ethnobotanically directed and chemotaxonomic selection has proven effective time and time again (Penna *et al*., 2001). The search for drugs and dietary supplements derived from plants has accelerated in recent years. WHO estimates that 80% of the world's population presently uses herbal medicine for some aspect of primary health care (WHO, 2003). A logical step in phytomedicine discovery is the screening of potentially bioactive plant extracts and combinations against a battery of tests to establish biological activity.

In search for novel drugs or an effective herbal combination against TB, three plants readily available in South Africa, were investigated for their combined antimycobacterial activity. *Euclea natalensis*, *Knowltonia vesicatoria* and *Pelargonium sidoides*. These three plants were selected based on their traditional uses (as stated in literature) for the treatment of tuberculosis, colds, flu or as immune boosters. *Euclea natalensis* is known to contain naphthoquinones which was found to exhibit significant antimycobacterial activity. *Pelargonium sidoides* has substantial evidence to support its immune enhancing properties. *Knowltonia vesicatoria* has not been researched as extensively as *E. natalenis* or *P. sidoides*. A detailed description of each plant selected for the present study is as follows:

### *2.2.1. Euclea natalensis* A.DC.

The Ebenaceae is a medium sized family comprising about 500 species but only two genera, *Euclea* and *Diospyros*, are found in the tropics and subtropics (Pooley, 1993). The roots of *Euclea* species are used in southern African traditional medicinal preparations to treat chest complaints, chronic asthma, leprosy and infections, among other ailments (Watt and Breyer-Brandwijk, 1962; Palgrave, 1977; Pujol, 1990; Hutchings *et al*., 1996).

### 2.2.1.1. Description and distribution

*Euclea natalensis* (Fig. 2.1.a.) is a shrub or small to medium sized tree with a spreading crown. The leathery leaves (glossy green above and woolly hairs below) are elliptic to oblong with a wavy margin. The small, sweetly scented flowers are greenish to white cream, borne as densely branched axillary heads. The edible berries are red and turn black as they mature (van Wyk and van Wyk, 1997). *Euclea natalensis* is widely distributed in tropical, subtropical Africa and is common on the East coast of South Africa, occurring in a wide variety of habitats, including coastal and inland forests as well as bushveld (Fig. 2.1.b; van Wyk and van Wyk, 1997).

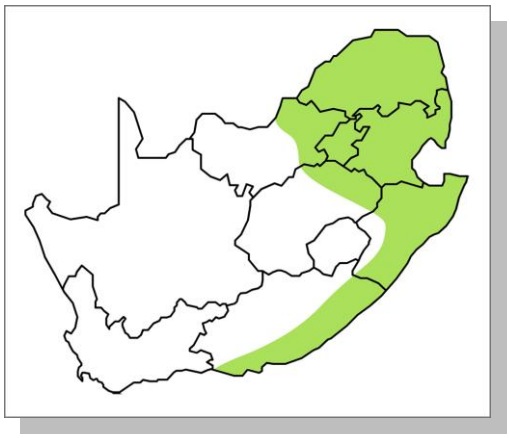
**(a)**  **(b)** 

**Fig. 2.1. (a) *Euclea natalensis* (www.worldbotanical.com) (b) Distribution of *E. natalensis* in South Africa**

### 2.2.1.2. Medicinal uses

The Zulu and Shangaan tribes use roots of this tree for curing different forms of chest complaints such as bronchitis, pleurisy and chronic asthma. The custom of cleaning teeth and gums with the charred roots of *E. natalensis*, in the belief that it benefits oral health, is practiced in South Africa by married women of the Zanzibari community. Charred and powdered roots are used as a treatment for ancylostomiasis and applied to skin lesions in leprosy (Bryant, 1966; Watt and Breyer-Brandwijk, 1962). The powdered roots are also used to relieve headache and toothache by local inhabitants of South Africa (Palgrave and Drummond, 1997).

### 2.2.1.3. Phytochemistry

Several secondary metabolites such as triterpenoids and naphthoquinones have been isolated from *E. natalensis* (Tannock, 1973; Khan and Rwekika, 1992; Van der Vijver and Gerritsma, 1974; Ferreira *et al*., 1977; Lall and Meyer, 2001). Nine of these compounds are naphthoquinones, including 7-methyljuglone (Fig. 2.3.b.), diospyrin, neodiospyrin and isodiospyrin (Van der Kooy *et al*., 2006). Other compounds include dihydroxyursanoic acids (lactone derivatives), and one tetrahydroxyflavanone arabinopyranoside (Van der Kooy *et al*., 2006). Shinanolone,

octahydroeuclein and 20(29)-lupene-3β-isoferulate, lupeol and betulin, have been isolated as inactive constituents (Weigenand *et al*., 2004).

#### 2.2.1.4. Antimycobacterial activity

The antimycobacterial activity against *M. tuberculosis* by extracts of *Euclea natalensis* roots has been previously reported (Lall and Meyer, 1999). The isolated compound shinanolone exhibited antimycobacterial activity against *M. tuberculosis* (with an MIC of 100 µg/ml), and antibacterial activity against a variety of test organisms (Weigenand *et al*., 2004). In further studies of the intracellular activity of naphthoquinones and triterpenes isolated from *E. natalensis* roots, it was established that the naphthoquinone 7-MJ has superior intracellular (in a macrophage cell line) and extracellular inhibition of *M. tuberculosis* relative to the anti-TB drugs streptomycin and ethambutol (Lall, *et al*., 2005). Diospyrin and 7-methyljuglone exhibited MICs of 8.0 and 0.5 µg/ml respectively against drug-sensitive *M. tuberculosis* (Lall *et al*., 2005). The compound 7-MJ was shown to synergistically enhance the antitubercular activity of isoniazid and rifampicin both extracellularly and intracellularly (Bapela *et al*., 2006). Another two naphthoquinones, neodiospyrin and isodiospyrin were also isolated from *E. natalensis* roots with activity against *M. tuberculosis*. The MIC value for both isodiospyrin and neodiospyrin was 10.0 µg/ml (Van der Kooy *et al*., 2006).

##### *2.2.1.4.1. Activity on the flavoprotein disulfide reductase (FDR) enzyme family*

Naphthoquinones are well known to operate as subversive substrates with flavoprotein disulfide (FAD) reductases such as glutathione reductase (Gtr), trypanothione reductase and lipoamide dehydrogenase (Salmon-Chemin *et al*., 2001 and Biot *et al*., 2004). Even as early as 1963 (Kusonose and Goldman) it has been demonstrated that naphthoquinones are reduced by a particular enzyme system, obtained from cell-free extracts of *Mycobacterium tuberculosis*. Thirty-five years later (Patel and Blanchard, 1999) this enzyme system was discovered to be that of Mycothione reductase (Mtr) which sustains millimolar concentrations of a structurally distinct low molecular thiol, Mycothiol (MSH) in lieu of glutathiol (GSH). Even though this mycobacterial thiol has a structure very different to GSH, it has similar functional roles in protection against oxidative stress derived from basal metabolic activities as well as from host-parasite confrontation (Patel and Blanchard, 1999). MSH is essential for the growth of *M. tuberculosis* (Sareen *et al*., 2003) and MSH-deficient mycobacteria exhibit increased sensitivity to oxidative stress (Rawat *et al*., 2002 and Newton *et al*., 1999).

The biological importance of Mtr and its ability to turnover other naphthoquinone substrates prompted a study of the substrate properties of naphthoquinones isolated from *E. natalensis* (Mahapatra *et al*., 2007). Mahapatra *et al* (2007) confirmed that these naphthoquinones serve as subversive substrates for Mtr, a consequence of their enzymatic reduction to semiquinone radicals (Fig. 2.2.). The naphthoquinone is regenerated via the concomitant reduction of oxygen to toxic

superoxide anion radicals. In this manner the naphthoquinone substrate is regenerated and the futile redox cycle continues. Naphthoquinones impair crucial metabolic or signalling pathways by causing the oxidation or inactivation of nucleophilic sites in signalling proteins of *M. tb*.

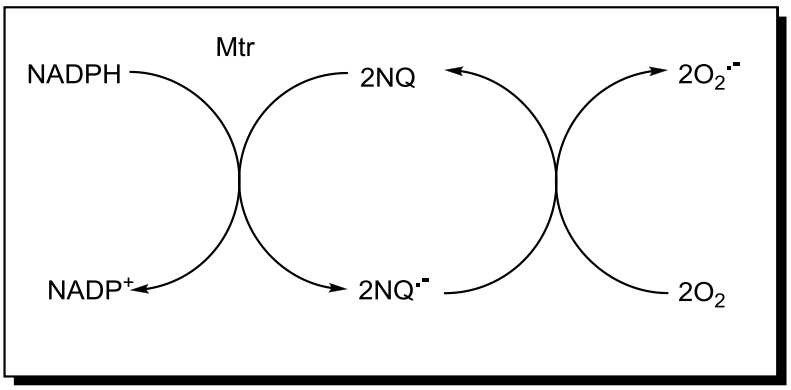



**Fig. 2.2. The futile enzymatic redox cycle of naphthoquinones (Mahapatra *et al*., 2007)**

### 2.2.1.5. Immunomodulatory properties

To date no literature on the immune modulating activity of *Euclea natalensis* has been found. In general there is very little information concerning immunomodulation for the Ebenaceae genus. Recent studies (Zhang *et al*., 2010) reported on the immune activity of a closely related species, *Diospyros kaki* (persimmon fruit). Although the activity of this plant can mostly be attributed to the high polysaccharide content of the fruit as it is well documented that plant derived polysaccharides have immune modulating effects (Capek and Hříbalová, 2004; Hokputsa *et al*., 2004; Popov *et al*., 2005) by stimulating cellular and humoral immune responses (Jiao *et al*., 2009; Schepetkin and Quinn, 2006).

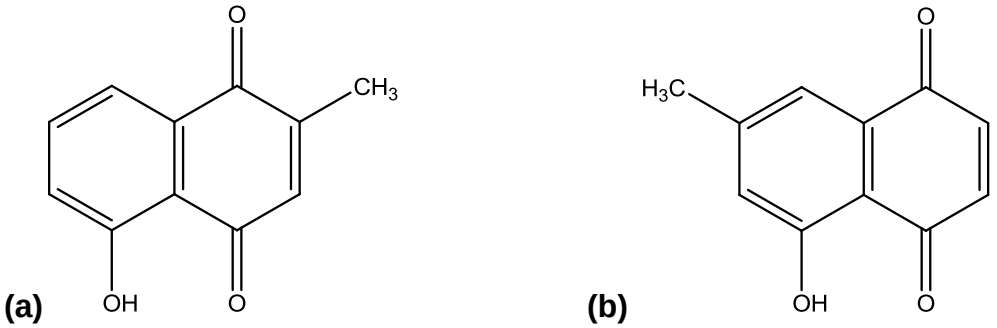



**Fig. 2.3. (a) Plumbagin (b) 7-methyljuglone**

Plumbagin (Fig. 2.3.a.), a naphthoquinone similar in structure to the naphthoquinones known to be present in *Euclea natalensis* (such as 7-MJ; Fig. 2.3.b.) has been reported to inhibit NF-κB (Sandur *et al*., 2006), induce apoptosis in different cell types (Jaiswal *et al*., 2002; Srinivas *et al*., 2004; Devi *et al*., 1998) and inhibit inflammation. It was found that similar to plumbagin, shikonin also inhibited activation of NF-κB pathway by tumour necrosis factor (TNF; Min, 2008). Another related

compound, β-lapachone (a lipophilic *o*-naphthoquinone), is known to mediate immune modulating activities (Kreher *et al*., 1988). Queiroz *et al*. (2008) recently described the antitumour effects of β-lapachone, which is suggested to result from enhanced macrophage activation against Ehrlich ascites tumour cells by acting synergistically with cytokines such as TNF-α.

### *2.2.2. Knowltonia vesicatoria* (L.f.) Sims

The Ranunculaceae comprises 59 genera and approximately 1900 species. Recently the genus *Knowltonia* was subsumed within the closely related genus *Anemone* and *Knowltonia vesicatoria* is now known as *Anemone vesicatoria* (L.f) Prantl (Ehrendorfer *et al*., 2009 and Manning *et al*., 2009). Throughout the dissertation the genus name *Knowltonia* has been retained. The genus *Knowltonia* (Ranunculaceae) is endemic to South Africa. There are at least ten species native to South Africa and an uncertain number growing in the Amazon Basin while there are now four species of *Anemone* found in South Africa (www.zipcodezoo.com).

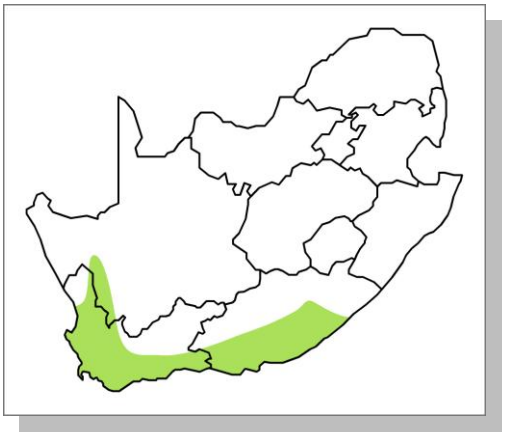
**(a)**  **(b)** 

**Fig. 2.4. (a) *Knowltonia vesicatoria* (b) Distribution of *K. vesicatoria* in South Africa (van Wyk *et al*., 1997)**

#### 2.2.2.1. Description and distribution

*Knowltonia vesicatoria* (Ranunculaceae, Fig. 2.4.a), also known as Blisterleaf or *Brandblaar* (in Afrikaans), is a perennial herb with a tuft of leathery, dark green leaves, the stems can grow up to 1.2 m tall. The leaves are divided into three leaflets and attached with leaf stalks to a short horizontal rootstock (rhizome) that is firmly anchored by fleshy roots. The small, white or yellowish to green flowers are borne in clusters just above the foliage, mainly in spring (Aug-Oct) and have no petals, only the greenish to white coloured sepals. The flowers are followed by fleshy fruits that turn black as they ripen (van Wyk *et al*., 1997). *Knowltonia vesicatoria* occurs in the southern parts of South Africa (Fig. 2.4.b), mainly the Western Cape. It is widespread within the fynbos of the Cape, from the Bokkenveld Mountains near Nieuwoudtville, south to the Cape Peninsula and eastwards to Knysna, Port Elizabeth and Grahamstown.

### 2.2.2.2. Medicinal uses

There are a large number of reported uses of *Knowltonia* and *Anemone* plants in folk medicine (Campbell *et al*., 1979). The species name *vesicatoria* is from the Latin *vesicare* which means to raise blisters, referring to the strong allergic reaction caused by the leaves and roots. The Afrikaans common name *Brandblaar* also refers to this allergic skin reaction. The early settlers at the Cape recognised *Knowltonia* as similar to European herbs such as *Helleborus* and it quickly became a popular herb with numerous uses. The leaves were used as a plaster over aching backs and joints. The burning sensation, often causing blisters, was considered beneficial for treating arthritis and rheumatism. Colds and flu were treated with a tea made from the roots mixed with *Pelargonium* roots. Toothache was alleviated by placing a piece of root into the cavity of a decaying tooth (van Wyk *et al*., 1997).

### 2.2.2.3. Phytochemistry

The Ranunculaceae family is known to contain many triterpenoid saponins and cyanogenesis also occurs in some of the species (Takhtajan, 2009). Most species of the Ranunculaceae family contain the toxic irritant oil known as protoanemonin (Fig. 2.5.a.). Protoanemonin, a hemiterpenoid lactone (or a gamma-hydroxyvinylacrylic acid lactone) is responsible for the allergic contact dermatitis induced by various Ranunculaceae and this oil is the active compound which causes the skin blistering of *K. vesicatoria*. Protoanemonin has been known to be the active antimicrobial compound of plant species of *Ranunculus bulbosus*, (Mares, 1987), *Anemone pulsatilla* (Baer *et al*., 1945; Cavallito and Haskell, 1945), and *Clematis dioscoreifolia* (Herz *et al*., 1951). When protoanemonin is dried the compound cyclodimerizes to cyclobutane-1,2-diol-1,2-diacrylic acid (Fig. 2.5.b.) and then to its non-toxic dilactone, called anemonin (van Wyk *et al*., 1997 and Bruneton, 1995; Fig. 2.5.c.).

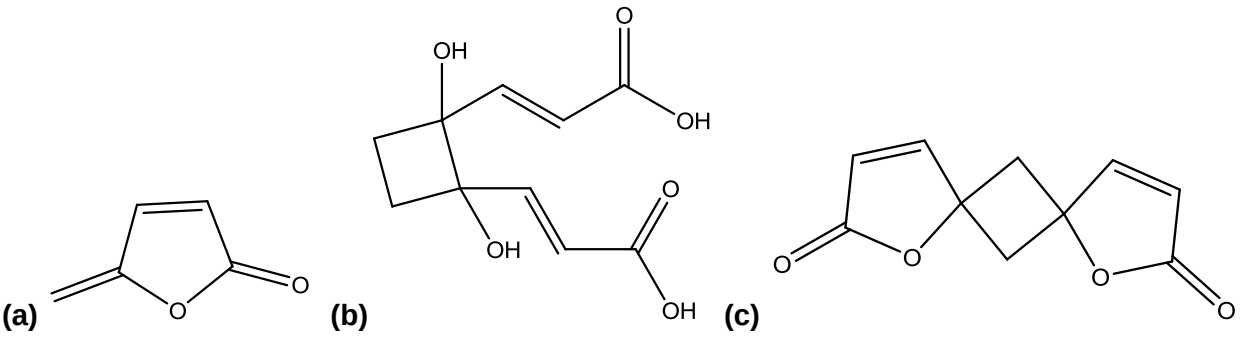



**Fig. 2.5. (a) Protoanemonin and (b) cyclobutane-1,2-diol-1,2-diacrylic acid and (c) anemonin (van Wyk, 1997)**

Anemonin, protoanemonin and ranunculin have been isolated from *Knowltonia capensis* (Campbell *et al*., 1979). It was found that anemonin was the compound present in the aqueous and ethanol extracts of fresh *K. capensis* which exhibited significant antibacterial activity against

*Staphylococcus aureus*, and significant *in vivo* antileukemic activity in the P388 lymphocytic leukemia test system (Campbell *et al*., 1979). Steam distillation of fresh plant material and extraction of the steam distillate with chloroform afforded protoanemonin, which rapidly polymerized to anemonin while extraction of fresh plant material with dilute HCl yielded the stable glucoside, ranunculin (Campbell *et al*., 1979).

#### 2.2.2.4. Antimycobacterial activity

Previous (unpublished) work suggests an MIC of 1250 µg/mL for the aerial methanol extract of *K. vesicatoria* (Nielsen, 2008) and an MIC of 625.0 µg/mL for the aerial ethanol extract (Labuschagné, 2008) against non-pathogenic *M. smegmatis*. The MIC against a sensitive strain of *M. tuberculosis* exhibited an MIC of 38.00 µg/mL and 50.00 µg/mL for the methanol and ethanol extracts, respectively.

##### *2.2.2.4.1. Activity on the FDR enzyme family*

The crude EtOH extract of *K. vesicatoria* was screened for possible inhibition or subversive substrate activity of both Mtr and Gtr. Even at the highest concentration tested (125.0 ug/mL), no significant inhibitory or subversive activity was observed (Labuschagné, 2008).

#### 2.2.2.5. Immunomodulatory properties

The most well known Ranunculaceae that is used as an immune-modulator is *Nigella sativa* (Black Seed, Black Cumin; van Wyk and Wink, 2004). It has been reported that black cumin stimulates bone marrow and immune cells, raises interferon (IFN) production, destroys tumour cells and protects against allergies and infections (Majdalawieh *et al*., 2010; Medenica, 1997; Schleicher and Saleh, 2000). The active compound, thymoquinone, inhibits allergic airway inflammation in a mouse model by inhibiting production of Th2 cytokines, inducing IFN-gamma and blocking eosinophil infiltration (El Gazzar *et al*., 2006). Immunological activity of the EtOH extract of *K. vesicatoria* aerial parts was attempted via flow cytometry by measuring cytokine levels (IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α) of TB-infected U937 macrophages treated with this extract (Labuschagné, 2008). The results indicated a mixed Th1/Th2 immune response at a concentration of 200 µg/ml.

### *2.2.3. Pelargonium sidoides* DC.

The family Geraniaceae consists of five genera; *Geranium, Erodium, Monsonia, Sarcocaulon* and *Pelargonium*. The genus *Pelargonium* comprises approximately 270 distinct species of perennial small shrubs of which about 80% occur in southern Africa with the centre of diversity in the Cape Province (Van der Walt and Vorster, 1988).

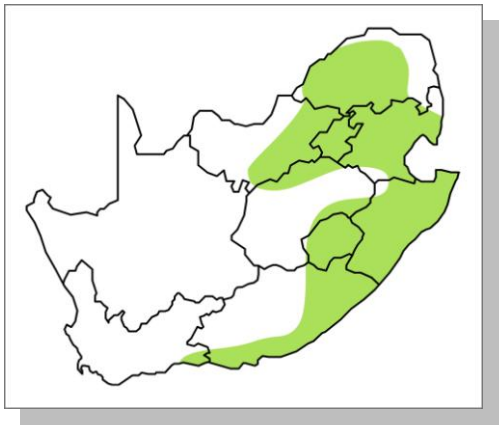
(a)  (b) 

**Fig. 2.6. (a) *Pelargonium sidoides* (Brendler and van Wyk, 2008) (b) Distribution of *P. sidoides* in South Africa**

### 2.2.3.1. Description and distribution

In its unadulterated form, *Pelargonium sidoides* (Fig. 2.6.a.) can readily be distinguished by the cordate shape of the leaves and the dark, maroon-red to black petals of the flowers borne on long, slender stalks (Van der Walt and Vorster, 1988). *Pelargonium sidoides*, also known as 'Kalwerbossie' is predominantly found over large parts of the interior of southern Africa (Fig. 2.6.b.), including the Eastern Cape, Free State, Southern Gauteng, South-western Gauteng and Lesotho, but also occurs in coastal mountain ranges (Van der Walt and Vorster, 1983; 1988).

### 2.2.3.2. Medicinal uses

*Pelargoniums* are important traditional medicines in South Africa. These plants, often referred to by their original Khoi-Khoi name *rabas* or *rabassam*, were amongst the first to be recorded by early explorers (Brendler and van Wyk, 2008). The fleshy, bright red tubers or rhizomes have been widely used by different cultural groups, mainly to treat diarrhoea and dysentery. In addition, *Pelargonium sidoides* is traditionally used to treats colds and flu in combination with roots of *K. vesicatoria* (van Wyk *et al*., 1997). Xhosa people in Transkei use *P. sidoides* to treat a prolapsed rectum, severe diarrhoea and a stomach ailment in babies known as *intisila*. In the Grahamstown region it has similar uses for the treatment of diarrhoea, a prolapsed rectum and *intisila*, in addition it is also used as a gripe water for infants and as a treatment for gonorrhoea. The plant is a useful astringent in dysentery. The roots are used by Zulu people to treat a sore throat, it has been reported to be used to treat tuberculosis (Brendler and van Wyk, 2008). *Pelargonium sidoides* has been developed into a highly successful, evidence-based phytomedicine with the most widely known herbal preparation or proprietary extract known as *Umckaloabo* or EPs® 7630, a popular remedy in Germany. In South Africa there are a few *P. sidoides* preparations including Linctagon (Fig. 2.7.).


**Fig. 2.7. Linctagon, a popular South African *P. sidoides* preparation**

### 2.2.3.3. Phytochemistry

Although these plants have a long tradition as ornamental and medicinal plants (Lis-Balchin, 2002), limited chemical sampling of members of this genus produced mainly common organic acids, derivatives of cinnamic acid, flavonoids, tannins, some coumarins and phytosterols (Williams and Harborne, 2002). With the exception of the detection of the unique alkaloids (epi)elaeocarpidin in hybrids (Lis-Balchin *et al*., 1996), all recent papers deal with these types of secondary products. The root extract of *P. sidoides* contains a wealth of highly oxygenated simple coumarins, unique in its composition (Table. 2.2.). For example, 6,8-dihydroxy-5,7-dimethoxycoumarin and 7-acetoxy-5,6-dimethoxycoumarin represent new natural products; the latter being the first natural compound known hitherto within this group possessing an acetoxy function (Kolodziej, 2007). As for the exclusive 7-hydroxy-5,6-dimethoxycoumarin (umckalin) and 5,6,7-trimethoxycoumarin, earlier work has documented their natural occurrence by their isolation from *P. reniforme* (Wagner and Bladt, 1974; 1975), which, however, is subject to taxonomic ambiguity. Noteworthy is also the characterization of three novel coumarin sulphates, obtained from polar fractions (Latté *et al*., 2000). This group of analogues has been restricted to three examples reported from a single plant source, *Seseli libanotis* (Apiaceae; Lemmich and Shabana, 1984). Extensive chromatographic efforts by Kolodziej (2007) have additionally yielded three coumarin glycosides in traces.

In the aerial parts, proanthocyanidins were again associated with members of hydrolysable tannins, as evidenced by the identification of brevifolincarboxyclic acid and corilagin. Additional notes concern the very limited occurrence of coumarins, in contrast to the abundance in the roots, the distinct presence of *C*-glycosyl flavones including members of the exclusive series of 2''-*O*-galloyl analogues (vide supra) and the new metabolite, 4-allyl-2,5-dimethoxyphenol 1-β-D-glucoside (Gödecke, 2005). The identification of 6,7-dihydroxycoumarin-8-sulfate not only extends

the range of unique natural coumarin sulphates, but also introduces the first member found in the aerial parts (Gödecke *et al*., 2005).

**Table. 2.2. Constituents of the root material of *P. sidoides* (Kolodziej, 2007).**

| **Compounds** |
|---|
| *Phenolic acids, phenylpropanoids and derivatives* |
| Gallic acid |
| Gallic acid methyl ester |
| Shikimic acid 3-*O*-gallate |
| |
| *Coumarins* |
| 7-Hydroxy-6-methoxycoumarin (Scopoletin) |
| 7-Hydroxy-5,6-dimethoxycoumarin (Umckalin) |
| 7-Acetoxy-5,6-dimethoxycoumarin |
| 5,6,7-Trimethoxycoumarin |
| 6,7,8-Trihydroxycoumarin |
| 6,8-Dihydroxy-7-methoxycoumarin |
| 7,8-Dihydroxy-6-methoxycoumarin (Fraxetin) |
| 6,8-Dihydroxy-5,7-dimethoxycoumarin |
| 5,6,7,8-Tetramethoxycoumarin (Artelin) |
| 8-Hydroxy-5,6,7-trimethoxycoumarin |
| |
| *Coumarin glycosides* |
| Magnolioside |
| Isofraxoside |
| Umckalin-7- β-D-glucoside |
| |
| *Coumarin sulfates* |
| 5,6-Dimethoxycoumarin-7-sulfate |
| 6,7-Dihydroxycoumarin-8-sulfate |
| 6-Hydroxy-5,7-dimethoxycoumarin-8-sulfate |
| 8-Hydroxy-5,7-dimethoxycoumarin-6-sulfate |
| |
| *Flavan-3-ols/Proanthocyanidins* |
| Catechin |
| Gallocatechin |
| Proanthocyanidins |
| |
| *Miscellaneous* |
| β-Sitosterol |

#### 2.2.3.4. Antimycobacterial activity

The biological activity of *P. sidoides* has been discussed extensively by Brendler and van Wyk (2008). Taylor (2003a, b) as well as Seidel and Taylor (2004) established antimycobacterial activity for hexane extracts of roots of *Pelargonium reniforme* and *Pelargonium sidoides*. They claimed that several mono- and diunsaturated fatty acids are the active compounds (with oleic acid and linoleic acid being considered the most active, having MICs of approximately 2.0 g/ml). Gödecke (2005) tested extracts and fractions of *Pelargonium sidoides* against two strains of Mycobacteria. Since no significant effect on the bacterial growth could be shown, it was assumed that the effective use of the plant in tubercular conditions may be due to an activation of the immune

system. This assumption was supported by Mativandlela *et al*. (2006, 2007), who investigated various extracts and isolated compounds from *Pelargonium sidoides* root with regards to their antimicrobial and especially their antimycobacterial activities. Strains of *Moraxella catarrhalis*, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium oxysporum, Haemophilus influenza, Mycobacterium tuberculosis* and *M. smegmatis* were exposed to acetone and ethanol root extracts, as well as four coumarins and two flavonoids isolated from *Pelargonium sidoides*. Significant activity could be shown for ethanol extract against *Aspergillus niger* and *Fusarium oxysporum* but limited activity against *Rhizopus stolonifer* and *Mycobacterium tuberculosis*. None of the isolated compounds showed any activity against *Mycobacterium tuberculosis*.

*2.2.3.4.1. Activity on the FDR enzyme family*

There is no known information on any mammalian FDR activity of *Pelargonium*. Some flavanoids have been shown to inhibit Gtr activity (Elliott *et al*., 1992; Khushbaktova *et al*., 1991). Different classes of polyphenols vary in their potency of Gtr inhibition in the following order: chalcones>tannic acid>flavonoids>coumarins>catechins (Zhang *et al*., 1997). As illustrated above (section 2.2.3.3.) the roots of *Pelargonium sidoides* contain a wealth of phenolic compounds including phenolic acids such as Shikimic acid 3-*O*-gallate, numerous coumarins and flavanols such as catechin.

**2.2.3.5. Immunomodulatory properties**

Kayser *et al*. (1997, 2001, 2003) investigated extracts and isolated constituents of *Pelargonium sidoides* for their effects on non-specific immune functions in different bioassays. All the extracts and compounds significantly reduced the intracellular survival of *Leishmania donovani*. This implies the activation of leishmanicidal macrophage functions. Activation was confirmed through the presence of tumour necrosis factor alpha (TNF-α) and inorganic nitric oxides (iNO). Synthesis of the latter is a known mechanism of macrophages against microorganisms.

Kolodziej *et al*. (2003) and Janecki *et al*. (2007) observed TNF inducing potencies for an extract of *Pelargonium* (EPs® 7630) as well as interferon-like activities in supernatants of sample-activated bone marrow-derived macrophages in numerous functional assays. Various subfractions of EPs® 7630 were tested for their NO-, TNF- and interleukin (IL)-12-inducing capacity. EPs® 7630 induced significant TNF levels in non-infected and GFP-transfected-*Leishmania major*-infected macrophages. Production of NO and IL-12, however, were negligible, while flow cytometry indicated a decrease in parasites in cells treated with EPs® 7630. This suggests that radical scavengers or low but efficient NO levels may be present in EPs® 7630.

Koch *et al*. (2002) further investigated if and how Umckaloabo interferes with interferon-beta (IFN-β) synthesis in MG-63 human osteosarcoma cells. IFN-β production increased in cells pre

incubated with Umckaloabo. Enhancement of natural killer cell mediated cytotoxicity was also found. Umckaloabo thus enhanced but did not induce IFN-β production.

Kolodziej *et al*. (1999, 2005) investigated polyphenol-containing extracts of *Pelargonium sidoides* and simple phenols, flavan-3-ols, proanthocyanidins and hydrolysable tannins for gene expressions (inducible Nitric Oxide Synthase or iNOS, IL-1, IL-10, IL-12, IL-18, TNF-α, IFN-α/γ). All extracts and compounds were capable of enhancing the iNOS and cytokine mRNA levels in parasitised cells.

Trun *et al*. (2006) carried out gene expression analyses using the reverse transcriptionpolymerase chain reaction for the iNOS and the cytokines IL-1, IL-12, IL-18, TNF-α, IFN-α, and IFN-γ in non-infected and in *Leishmania major*-infected RAW 264.7 cells. EPs® 7630 induced low mRNA levels in non-infected cells, but considerably up-regulated transcript expressions in infected cells. Production of IFN-γ mRNA was also stimulated. Similar profiles were obtained for the methanol-insoluble fraction (MIF) and gallic acid. The methanol-soluble fraction and umckalin did not show any significant gene-inducing capabilities. These results indicate that the inducing principle may be located in the MIF.

Kolodziej and Kiderlen (2007) investigated effects on non-specific immune functions by EPs® 7630, extracts and isolated constituents of *Pelargonium sidoides*. Significant immunomodulatory properties could be established in various functional bioassays. Gene expression experiments (iNOS, IFN-α, IFN-γ, TNF-α, IL-1, IL-10, IL12, IL-18) not only confirmed these data but also showed response differences of infected macrophages compared to non-infected cells.

Koch and Wohn (2007) investigated potential effects of EPs® 7630 on the release of antimicrobial peptides from neutrophils. The neutrophil granulocyte, containing antimicrobial peptides and proteins with a wide range of antimicrobial as well as chemotactic, immunomodulating and wound healing activity (such as bactericidal/permeability-increasing protein [BPI] and defensins), is a key cellular component of immune response. EPs® 7630 at concentrations between 0.3 and 30µg/ml was added to heparinized whole human blood samples. Analysis of plasma for BPI and human neutrophil peptides (HNP) 1–3 content after 5 h incubation revealed a significant dose-dependent increase in the release of HNP 1–3 and BPI. Thus EPs® 7630 seem to stimulate host defence through enhancing the release of antimicrobial peptides.

## 2.3. Collection of plants

*Knowltonia vesicatoria* was bought at two different nurseries situated in Johannesburg. Aerial parts (stems and leaves) were collected, resulting in a net weight of approximately 1.96 Kg. Previously,

roots of *P. sidoides* were collected from Qwaqwa, a region in the Free State province of South Africa and roots from *E. natalensis* were collected from Tembe Elephant Park in KwaZulu-Natal province of South Africa. Voucher specimens were deposited and identified at the H.G.W.J Schweicherdt Herbarium (PRU) of University of Pretoria (Table. 2.3.).

**Table. 2.3. Plants selected for the present study**

| Scientific name (Family) | Common name | Plant parts used | Voucher specimen |
|---|---|---|---|
| *Euclea natalensis* A.DC. (Ebenaceae) | Gwharriebush | Root | N.L. 22 |
| *Knowltonia vesicatoria* (L.f.) Sims, now known as *Anemone vesicatoria* (L.f) Prantl (Ranunculaceae) | Blisterleaf, Brandblaar | Aerial | PRU 096449 |
| *Pelargonium sidoides* DC. (Geraniaceae) | Kalwerbossie, rabassam | Roots | PRU 092559 |

## 2.4. Materials and methods

### *2.4.1. Extraction of plant material*

The collected aerial parts of *K. vesicatoria* were allowed to air-dry in open sample bags away from direct sunlight. The dried plant material resulted in 530.0 g. For each 20.00 g of dried plant material, approximately 100 mL of EtOH was added. The solvent and plant material was placed in capped, dark-glass bottles (5L capacity) on an electric shaker. The closed bottles were vigorously shaken by hand twice a day. Each day, for a total of 7 days, the plant material was filtered, the extract concentrated under reduced pressure and clean EtOH added to the remaining plant material. A total yield of 148.4 g of dried extract was obtained (28% of the total dried plant material). Prepared extracts of *Euclea natalensis* (chloroform; $CHCl_3$) and *Pelargonium sidoides* (EtOH) was kindly donated by Prof. N. Lall.

### *2.4.2. Removal of tannins from the ethanol extract of K. vesicatoria*

Sixty grams of the *K. vesicatoria* EtOH extract was subjected to a solvent partitioning method for the removal of polyphenolics, described by Houghton and Raman (1998). The dried ethanol extract was dissolved in 1.5L of a 9:1 EtOH:$dH_2O$ solution which was then partitioned with an equal volume of hexane (Fig. 2.8.a.). The hexane layer was concentrated under reduced pressure and stored for further testing of the non-polar components (2.35 g). Half of the 90% ethanol extract (750 mL) was concentrated under reduced pressure and stored for further testing (11.88 g; extract still contains tannins but free from non-polar components). The remaining half of the ethanol layer (750 mL) was partitioned with equal volumes of chloroform:methanol (4:1) and water. The two

layers were then separated. The EtOH layer was concentrated under reduced pressure and stored (13.27 g). The chloroformic layer was then washed with an equal volume (750 mL) of 1% w/v NaCl in water. The chloroformic layer was then dried under reduced pressure, yielding 9.86 g of polyphenol-free extract.

Before tannin clean-up, the crude EtOH extract was tested for the presence of tannins by adding a few drops of Ferric chloride (5% $FeCl_3$ w/v in EtOH) which tested positive by producing a black green colour formation (Fig. 2.8.b.). After the tannin clean-up, the chloroformic layer/fraction tested negative for the presence of tannins (Fig. 2.8.c.). All five the different layers/fractions (Table. 2.4.) were screened for inhibitory activity against the non-pathogenic *Mycobacterium smegmatis*.

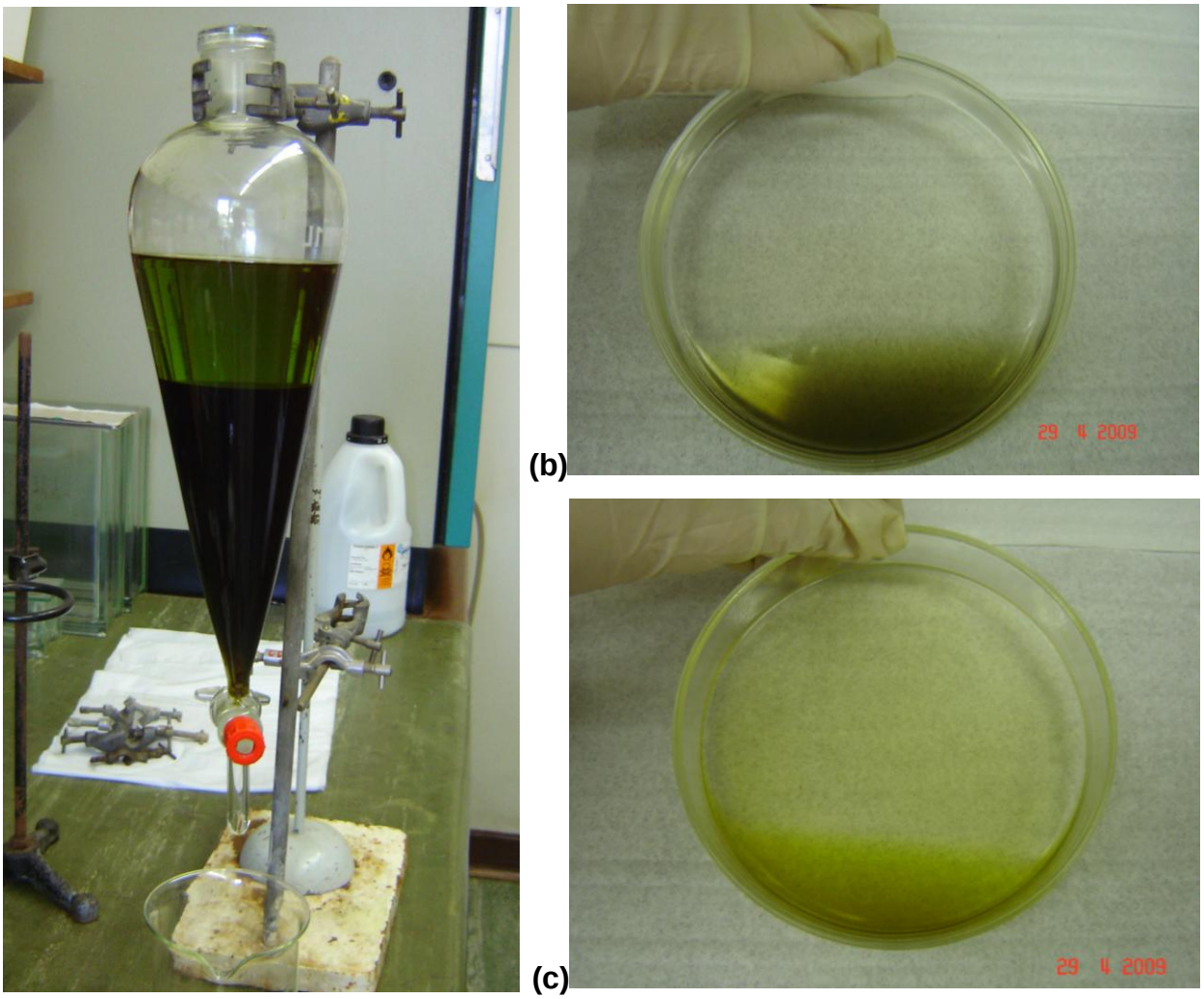



**Fig. 2.8. (a) Solvent partitioning. (b) Positive $FeCl_3$ detection test of crude EtOH *K. vesicatoria* extract for tannins (c) Negative $FeCl_3$ detection test of the extract after tannin removal.**

**Table. 2.4. Fractions obtained from solvent partitioning for tannin clean-up**

| Fraction | Solvent layer (yield) | Properties |
|---|---|---|
| F1 | First EtOH:d$H_2O$ [9:1] fraction (11.88 g) | Polar fraction, contains most crude constituents, no non-polar compounds |
| F2 | Hexane fraction (2.35 g) | Non-polar fraction, only contains non-polar constituents of crude extract |
| F3 | Second EtOH:d$H_2O$ [9:1] fraction (13.27 g) | Polar fraction, contains high concentration of polyphenols |
| F4 | $CHCl_3$ fraction (9.86 g) | Medium polarity, free from tannins |
| F5 | 1% NaCl solution (22.38g) | Salty layer, contains NaCl and polyphenols |

### *2.4.3. Antimycobacterial activity on M. smegmatis using microplate susceptibility testing*

#### 2.4.3.1. Test organism: *Mycobacterium smegmatis*

*Mycobacterium smegmatis* is an ideal organism to use as a surrogate for *M. tuberculosis* in biological assays as it is a fast growing, non-pathogenic organism that can be cultured in any laboratory in a liquid broth such as Middlebrook 7H9 and on media such as Middlebrook 7H11 agar. It is acid-fast staining and shares many features with the pathogenic *M*. *tuberculosis*, especially with regard to the cell envelope. These two organisms share many similar drug sensitivity profiles (Mitscher and Baker, 1998) and biosynthetic pathways (Ojha *et al*., 2000).

#### 2.4.3.2. Preparation of Micro-organisms

*Mycobacterium smegmatis* ($MC^2$ 155) cultures were obtained from American Type, MD, USA Culture Collection (ATCC) kindly donated from the Medical Research Council (MRC) in Pretoria, South Africa. The cultures were kept on Middlebrook 7H11 agar and stored at approximately 8°C, for no longer than one month, with a reference kept in cryovials at -70°C. To simplify the maintenance of the microorganism, a sufficient amount of growth was carefully scraped off from the cultures on one Petri dish, by using a sterile applicator stick and transferred into several sterile cryovials containing 3mL Middlebrook 7H9 broth and kept frozen at -70°C. Twenty four hours before the assay, colonies from two cryovials were left in a flow hood to defrost at room temperature and transferred into two sterile Erlenmeyer flasks containing 50mL of fresh Middlebrook 7H9 broth and incubated for 24hrs at 37°C. The overnight liquid culture was then transferred to a sterile test tube containing 20-25 glass beads (with a 2mm diameter) and homogenised by mixing on a Vortex mixer for 5-10min. The broth culture was then left still for 5-10min to let larger clumps of mycobacteria settle. The supernatant was then transferred to a new

test tube and 3mL decanted into a cuvette to be adjusted with 7H9 broth to reach an optical density (OD) of 0.2 log-phase at 550nm, measured by a spectrophotometer (Beckman Coulter DU® 720 UV/Vis), to ensure that the bacteria are at the start of the log growth phase for the experiment or rather the correct concentration of 1.26 x $10^8$ colony-forming units per millilitre (CFU/mL) (Salie *et al*., 1996 and Newton *et al*., 2002).

#### 2.4.3.3. Preparation of extracts

A stock concentration of 10.0 mg/mL of each sample (the crude *K. vesicatoria* EtOH extract and the five dried fractions obtained from solvent partitioning) was prepared in a solution of 10% dimethyl sulphoxide (DMSO) in sterile Middlebrook 7H9 broth (Sigma-Aldrich, South Africa). To ensure optimum dissolution of the extracts, the preparations were mixed on a Vortex mixer and sonicated at 25°C for 15 minute intervals until complete solution was achieved.

#### 2.4.3.4. The microdilution method

All samples were tested against *M. smegmatis* using the 96-well microtitre plate dilution method (Newton *et al*., 2002). The minimum inhibitory concentration (MIC) and the bactericidal effect (minimum bactericidal concentration, MBC) were determined according to the methods described by Salie *et al*. (1996). In short, 200 µL of distilled water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. The 96-well plates received 100 µL of Middlebrook 7H9 broth (Sigma-Aldrich, South Africa) and a serial dilution of the samples was made directly on the plate. The final sample concentrations tested were 2.50 to 0.08 mg/mL. Ciprofloxacin, at final concentrations ranging from 2.00x$10^{-3}$ to 7.80x$10^{-5}$ mg/mL, served as the positive drug control. Since none of the samples in the assay ever exceeded a DMSO concentration of more than 2.5% v/v, DMSO was added in duplicate, at final concentrations ranging from 50% to 1.56%, to serve as the solvent control. To give a final volume of 200.0 µL/well, 100.0 µL of *M. smegmatis* suspension (0.2 log-phase, yielding 1.26 x $10^8$ CFU/mL) was added to each well containing the samples and mixed thoroughly. One column of wells was reserved for 200.0 µL of broth only, to serve as a sterility control. The growth or negative control (in duplicate) consisted of 100.0 µL broth and 100.0 µL mycobacteria, each. The samples were tested in triplicate and the entire experiment was conducted twice. Plates were covered, sealed with parafilm and incubated at 37 °C for 24 h. After this time, 40 µL of a freshly prepared *p*-iodonitrotetrazolium chloride or INT (0.2 mg/mL; Sigma-Aldrich, South Africa) was added to the plate (excluding one column for each sample) and incubated at 37°C for 30 to 60 minutes (Eloff, 1998). A lack of pink colour in the wells was interpreted as no bacterial growth, and a pink colour was scored as growth. The MIC was defined as the lowest sample concentration, which prevented a colour change to pink.

The MBC was determined by adding 50.0 µL aliquots of the MIC preparations, all the concentrations higher and one lower than the determined MIC (from the initial INT excluded wells) to 150.0 µL of 7H9 broth in wells of a new 96-well plate. This new plate was covered with the lid, sealed with parafilm and incubated for 48hrs at 37°C. The MBC of these preparations was also detected by the addition of 40.0 µL INT (0.2 mg/mL) and incubated at 37°C for 30 to 60 minutes. The MBC was regarded as the lowest concentration of sample which did not produce the pink colour change. Extracts showing the lowest MIC and MBC results were screened against *M. tuberculosis* at MRC using the BACTEC drug susceptibility assay.

### *2.4.4. Determining synergistic antimycobacterial activity on M. tuberculosis using the BACTEC radiometric assay and a colorimetric assay*

#### 2.4.4.1. The BACTEC radiometric assay

Despite promising progress in the direct detection of tuberculosis by molecular biological methods, e.g., PCR or transcription-mediated amplification (Pfyffer *et al*., 1997), cultures still remain indispensable in the clinical mycobacteriology laboratory. This especially holds true with susceptibility testing which, when done routinely, requires ample biomass.

Radiometric respiratory with the BACTEC TB-460 system (Becton Dickinson Diagnostic Instrument, Sparks, md), is a well-documented technique for testing susceptibility of *M. tuberculosis* and has been used as described previously by other researchers (Lall and Meyer, 1999; Bapela 2005; Mativandlela *et al*., 2006). Although conventional cultivation is straightforward on solid medium such as Löwenstein-Jensen (LJ) or Middlebrook (7H10/7H11) agar, it is insensitive and may take several weeks (usually 3-5 weeks) to obtain results. The automated radiometric detection of *Mycobacterium* quantitatively determines susceptibility testing of *Mycobacterium* on the basis of the MIC of antibiotics. The BACTEC procedure for drug susceptibility testing for *Mycobacterium* is based on the same fundamental principle used in the conventional method. However, in the BACTEC radiometric assay a liquid medium (a $^{14}$C-labelled substrate enriched, 7H12 Middlebrook TB medium) contained in septum vials (12B vials; Fig. 2.9.a.) is used, and instead of counting colonies after three weeks, the growth can be monitored radiometrically and the results reported within 5 to 6 days. The accuracy and reproducibility of the BACTEC method has been evaluated and compared with the conventional and newer methods such as the BACTEC MGIT960 system (Becton Dickinson, Sparks MD; Fig. 2.9.b.) with outstanding results (Tarrand and Gröshel 1985; Pfyffer *et al*. 1997). The MGIT system uses Mycobacteria Growth Indicator Tubes (MGIT) these tubes have a silicon film embedded with a ruthenium salt at the bottom of the tube as a fluorescence indicator, fluorescence-emitting tubes are reported as positive for viable mycobacteria (Tortoli and Palomino, 2007). The BACTEC method is relatively labour intensive and holds other limitations such as high costs of acquisition of

the BACTEC system. The accumulation of radioactive waste and the potential danger of needle punctures among laboratory technicians are the most serious drawbacks. In addition to these disadvantages, the BACTEC system will soon become obsolete. Most BACTEC TB-460 machinery is as old as 20 years and Becton Dickinson has discontinued manufacture of the BACTEC 12B vials.

With the BACTEC method, drugs or plant extracts are incorporated in a $^{14}C$-labelled substrate (palmitic acid) enriched 7H12 Middlebrook TB medium. The organism uses the substrate and produces $^{14}CO_2$. The amount of $^{14}CO_2$ produced and measured reflects the rate and amount of mycobacteria growth, this is expressed as the growth index (GI) (Middlebrook *et al*., 1977). The critical proportion of resistance of *M*. *tuberculosis* is evaluated at the 1% level (Middlebrook *et al*., 1977).

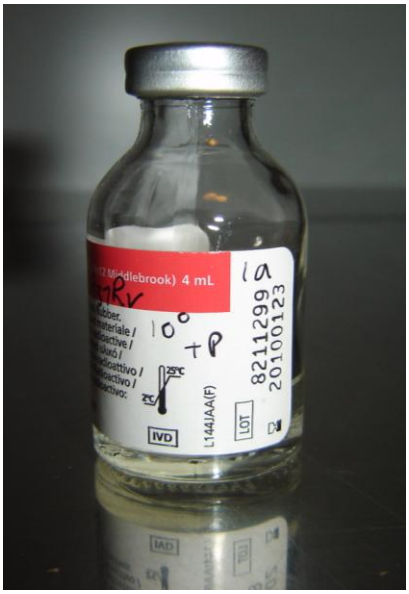
**(a)**  **(b)** 


**Fig. 2.9. (a) BACTEC 12B vial (b) BACTEC MGIT960 system**

### 2.4.4.2. Preparation of Micro-organisms

Three *M*. *tuberculosis* strains were used in the experimental procedures, which were carried out at the Medical Research Council (MRC), Pretoria, South Africa. A drug-susceptible strain of *M. tuberculosis* (sensitive to the first-line antituberculous drugs; INH, RIF, EMB and STR), H37Rv (ATCC 27294) was obtained from the American Type, MD, USA Culture Collection. *Mycobacterium tuberculosis* was plated onto slants of Löwenstein-Jensen (LJ) medium and allowed to grow for 3 - 4 weeks at 37$^{o}$C. Two clinical drug resistant strains, 4388 (resistant to INH and EMB) and 5497 (resistant to RIF, INH, EMB, STR) were maintained in BACTEC 12B medium at 37$^{o}$C until a growth index (GI) of 400 was reached. Both resistant strains were obtained from an MRC proficiency testing (round 14) from Belgium. These resistant strains were only used to test the activity of the *K. vesicatoria* (EtOH) extract.

To prepare the assay inoculum of the sensitive H37Rv strain, a sterile applicator stick was used to take a representative amount of colonies from 3 week-old cultures growing on LJ medium. This sample was transferred to a sterile 16 x 125 mm screw capped tube containing six to eight glass beads (1-2 mm) and 3.0-4.0 mL of diluting fluid (0.1% Tween 80/saline solution.). The culture mixture was homogenised by mixing the tube on a Vortex mixer for 5-10 minutes. After 15min when the large particles had settled, the supernatant was transferred into a separate sterile test tube and more diluting fluid was added to adjust the homogenous culture mixture to McFarland no 1 turbidity standard (Youmans and Youmans, 1948). This homogenate is referred to as V0 or the $10^0$ inoculum, yielding 1 x $10^4$ to 1 x $10^5$ CFU/mL. For the two drug resistant strains, the 12B vials containing cultures with a GI of 400 were designated as the assay inoculums or V0.

#### 2.4.4.3. BACTEC rapid radiometric assay using *M. tuberculosis*

To confirm previously stated antimycobacterial activity of the extracts from *K. vesicatoria* (EtOH), *P. sidoides* (EtOH) and *E. natalensis* ($CHCl_3$) against a susceptible strain of *M. tb*, these extract were tested again. Previous results for *K. vesicatoria* show an MIC of 50.00 µg/mL against H37Rv (Labuschagné, 2008), and was tested here at three concentrations (100.0, 50.00 and 25.00µg/mL) in triplicate. This extract was also tested against two drug-resistant strains of *M. tb* at the same concentrations. According to Lall *et al*. (2005) the chloroform root extract of *E. natalensis* has an MIC of 8.00 µg/mL against susceptible *M. tb*. This extract was tested again at concentrations ranging from 16.00, 8.00 to 4.00 µg/mL in triplicate. The ethanol extract of *P. sidoides* exhibited an MIC above 5000 µg/mL (Mativandlela *et al*., 2006) and was tested at three concentrations (10000, 5000 and 2500 µg/mL) in triplicate.

The hexane fraction (F2) and the tannin-free fraction (F4) obtained from the tannin clean up partitioning (section 2.4.2.), were tested in triplicate at three concentrations (100.0, 50.00 and 25.00 µg/mL) based on the antimycobacterial activity of these samples against *M. smegmatis*. All the samples were dissolved in 100% DMSO to obtain stock concentrations that were subsequently serially diluted two-fold to give the three test concentrations. All the samples were dissolved in 100% DMSO to obtain the relevant stock concentrations. Subsequent dilutions were made in DMSO and added to BACTEC 12B vials containing 4 mL of 7H12 medium broth to achieve the desired final concentrations of the samples (in triplicate) as mentioned above. For every triplicate, two vials were supplemented with 100 µL antimicrobial PANTA solution (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) whilst the remaining vial received an equal amount of reconstituting fluid consisting of a poly-oxyethylene solution (Becton Dickinson and Company, Ferndale, South Africa; Fig. 2.10.).


**Fig. 2.10. PANTA microbial supplement and reconstituting fluid (poly-oxyethylene).**

Control experiments showed that a final concentration of DMSO (1%) in the medium had no adverse effect on the growth of *M. tuberculosis*. Isoniazid (Sigma-Aldrich, South Africa) was used as the positive drug control at a concentration of 0.2 µg/mL. A homogenous culture (100 µL of V0) of *M. tuberculosis* was inoculated in the vials containing the extracts as well as in the control vials (Heifets *et al*., 1985). Three extract-free vials were used as controls (medium + 1% DMSO): one vial (V1) was inoculated in the same way as the vials containing the extracts, and two vials (V2, referred to as the $10^{-2}$ inoculum) were inoculated with a 1:100 dilution of V0 to produce an initial concentration representing 1% of the bacterial population (1 x $10^2$ to 1 x $10^3$ CFU/mL). The MIC was defined as the lowest concentration of the extract that inhibited >99% of the bacterial population. *Mycobacterium* metabolise the $^{14}$C-labelled palmitic acid in the 7H12 medium and expire $^{14}CO_2$. The amount of $^{14}CO_2$, detected by the BACTEC 460 system, reflects the rate and amount of growth that occurred in the sealed 12B vials and is expressed in terms of the growth index (GI; Middlebrook *et al*., 1977). Inoculated bottles were incubated at 37°C and each bottle was assayed at 24 h intervals to measure the GI, at approximately the same hour (± 2 hours) until cumulative results were interpretable. The difference in the GI values of the last two days is designated as ΔGI. The GI readings of the vials containing the test extracts were compared with the control vials (V2). Readings were taken until the control vials, containing a hundred times lower dilution of the inoculum than the test vials, reached a GI of 30 or more. If the ΔGI values of the vials containing the test extracts were less than the control vials, the population was reported to be susceptible to the compound and if the value was equal or greater than that in V2, the test organisms were considered to be resistant to the compound.

All procedures involving the transfer of *M. tb* cultures were carried out in a biological safety cabinet (Level 3; Fig. 2.11.) and the vial tops were wiped with 5% alcohol swabs before removal from the hood. Whenever results suggested contamination (e.g., large, rapid increase in GI), the bottles were inspected, Ziehl-Neelsen staining was carried out, a rapid protein “stick test” and 100 µL of the suspected vial medium was streaked on blood agar plates (Fig. 2.12.) to determine whether the visible microbial growth was organisms other than *Mycobacterium*.

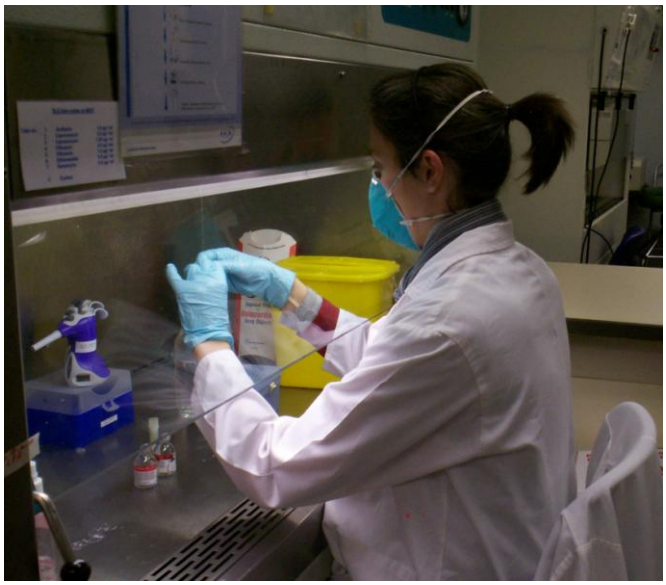
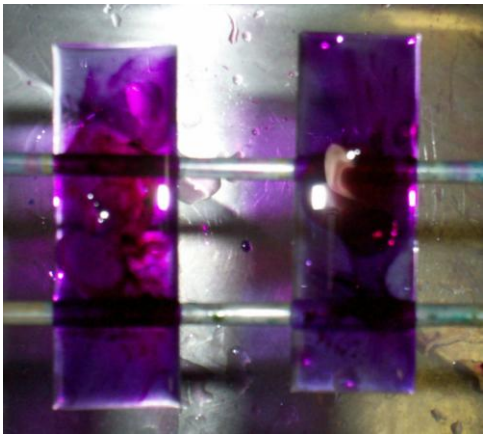
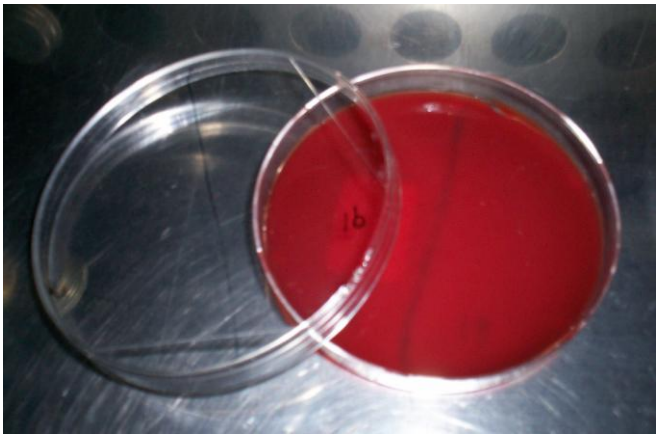


**Fig. 2.11. Laboratory with Level 3 biological safety cabinets at the Medical Research Council, Pretoria, South Africa.**

(a)  (b) 

(c) 

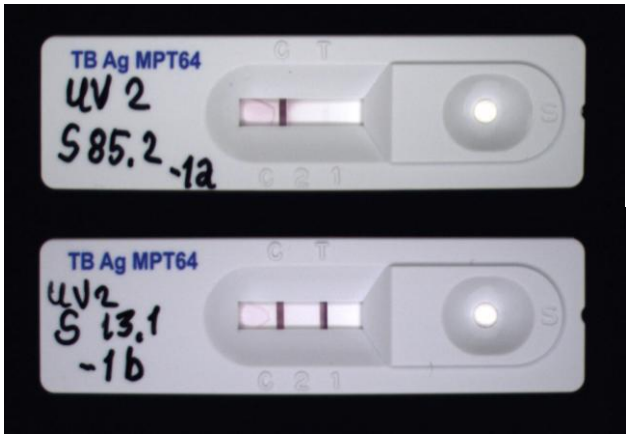




**Fig. 2.12. (a) Ziehl-Neelsen staining of two samples. (b) Blood agar plate- no contamination evident (c) Rapid TB Ag MPT64 test, two lines indicate positive for *M. tb* and one line indicates organisms other than *M. tb*.**

#### 2.4.4.4. Synergistic antimycobacterial activity

The activity of three different drug and extract combinations was evaluated at sub-MIC levels (below original MIC values) so that each component (extract or drug) was present at concentrations corresponding to ½, ¼ and ⅛ of the MIC. Analysis of the drug combination data was achieved by calculating the fractional inhibitory concentration (FIC) index (Bapela *et al*., 2006; Berenbaum, 1978; De Logu *et al*., 2002) as follows: FIC = (MIC $_{\text{a combination}}$/ MIC $_{\text{a alone}}$) + ( MIC $_{\text{b combination}}$ + MIC $_{\text{b alone}}$). The FIC was interpreted as: FIC ≤ 0.5, synergistic activity; FIC = 1, indifference / additive activity; FIC ≥ 2 or more, antagonistic activity. Subscripts a and b represent the two different components in a two-drug combination. Three different combinations of plant extracts and INH were tested for possible synergistic activity against the drug susceptible *M. tb* strain. Combination 1 (C1) included the extracts of *E. natalensis* ($CHCl_3$), *K. vesicatoria* (EtOH) and *P. sidoides* (EtOH) in a four-drug combination with the first line drug INH. Combination 2 (C2) only included the three plant extracts in a three-drug combination. Combination 3 (C3) combined the *K. vesicatoria* (EtOH) extract and INH in a two-drug combination. The BACTEC radiometric method, as described before in section 2.4.4.3., was used to determine the synergistic activity of these different amalgamations.

#### 2.4.4.5. The direct colorimetric microdilution assay

In view of the future obsolescence of the BACTEC TB-460 system, a new method for determining antimycobacterial activity against *M. tb* has to be established. Culture-based techniques on solid media for susceptibility testing takes 3-7 weeks to give results due to the slow growth rate of *M. tuberculosis*. Liquid culture systems, including MGIT, enable laboratories to determine *Mycobacterium* susceptibility to first-line drugs within 1-2 weeks incubation time, although this system is expensive and ill suited for quantitative MIC determination. Different colour development methods have been described to detect antibiotic resistance of *M. tuberculosis* complex clinical strains, like the MABA/Alamar Blue assay (Collins and Franzblau, 1997; Palomino and Portaels, 1999; Shiloh *et al*., 1997) and tetrazolium salt assays such as MTT and TTC (Abate *et al*., 1998; Mohammadzadeh *et al*., 2006). To develop a simple, rapid and inexpensive microdilution plate method, INT was employed to evaluate the MIC's of previously tested samples for the *M. tb* H37Rv reference strain. The yellow dye, *p*-iodonitrotetrazolium chloride (INT) is reduced in living cells by dehydrogenase to produce insoluble bright pink to purple INT formazan crystals (Denizot and Lang, 1986). This dye has long been in use in our laboratory for the detection of viable *Mycobacteria smegmatis*.

Briefly, 200 µL of sterile water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. The 96-plates received 100 µL of OADC (Oleic acid, albumin, dextrose and catalase; Sigma-Aldrich, South Africa) enriched Middlebrook 7H9 broth (Sigma-Aldrich, South Africa) and a serial dilution of the samples

was made directly on the plate. The final sample concentrations tested in triplicate, were 1.60-0.05 µg/mL for INH; 200-6.25 µg/mL for the *K. vesicatoria* (EtOH); 16.0-0.5 µg/mL for *E. natalensis* and 12.5-0.39 µg/mL for C3. An inoculum of 2 x$10^5$ CFU/mL was prepared by diluting a *M. tb* homogenate with equal turbidity to the McFarland no 1 standard (section 2.4.3.2) and 100 uL was added to the appropriate wells to yield a concentration of 1 x$10^5$ CFU/mL *M. tb* in the assay wells. Plates were covered, sealed with parafilm and incubated in a $CO_2$ (5%) incubator at 37 °C for 5 days. After this time, 40 µL of a freshly prepared INT (0.2 mg/mL) was added to one of the three *M. tb* growth control column wells and incubated for 24 h. If no pink colour appeared after 5 days, the plate was incubated for an additional 24h and the process repeated until a pink colour manifested. After the initial pink colour appeared in the growth control wells, newly prepared INT was added to the rest of the plate and incubated for a further 24 h before recording the results. A lack of pink colour in a test well was interpreted as no bacterial growth, and a pink colour was scored as growth. The MIC (minimal inhibition concentration) was defined as the lowest drug concentration, which prevented a colour change to pink.

## 2.5. Results and discussion

### *2.5.1. Antimycobacterial activity of solvent partitioned fractions on M. smegmatis using microplate susceptibility testing*

Although studies have shown that *M. smegmatis* could be more resistant to drug activity than *M. tuberculosis* (Tarrand and Gröschel, 1985; Mitscher and Baker, 1998; Zgoda and Porter, 2001), screening samples against non-pathogenic *M. smegmatis* gives quick results in any laboratory fitted for microbial tests and provides a good indication of which samples will also be active against *M. tuberculosis*. In most cases this strategy saves time and expensive reagents necessary to test numerous samples against the pathogen, *M. tb*. In this case, the purpose of screening the partitioned fractions was to promptly establish if the tannin-free extract still exhibits the same activity as the tannin containing extract. Results clearly indicate that this is not the case (Table. 2.5.). The tannin-free extract (F4) exhibited an MIC four-fold higher (2500 µg/mL) than that of the crude tannin containing EtOH extract (625.0 µg/mL). This does not automatically point to the conclusion that tannins are the responsible components for *Knowltonia*'s antimycobacterial activity. None of the partitioned fractions from *K. vesicatoria* exhibited an MIC or MBC as low as that of the crude or 'whole' EtOH extract. Fraction 1, which still contained all of the polyphenolic compounds but no non-polar compounds, had exactly the same activity profile as that of F4, with an MIC of 2500 µg/mL and an MBC of more than 2500 µg/mL. The highest activity seen for the fractions was that of the non-polar compounds (F2) with an MIC and MBC of 1250 and 2500 µg/mL, respectively. Fractions 3 (containing mostly polyphenols) and 5 (existing mainly of NaCl and polyphenol residue) had no activity at the highest concentration (2500 µg/mL) tested. The positive drug control, Ciprofloxacin exhibited an MIC equal to its MBC value of 1.250 µg/mL, which is comparable to its

mycobactericidal value found in literature (Inderlied, 2004). The highest percentage DMSO used for the samples in the assay (2.50%), did not inhibit *M. smegmatis* growth as evident with the MIC/MBC value of 12.50%. No contamination was present as no pink colour change occurred with any of the sterility controls. In all the growth control wells the Mycobacteria grew favourably with a bright pink colour change indicating viable bacilli. Taken as a whole, the results imply that either the active compounds were damaged during the partitioning procedure or that synergy forms the basis of the antimycobacterial activity of *K. vesicatoria*.

**Table. 2.5. MIC and MBC values of the solvent partitioned fractions against *M. smegmatis* compared to the crude EtOH extract of *K. vesicatoria***

| Sample | MIC[a] (µg/mL) | MBC[b] (µg/mL) |
|---|---|---|
| *K. vesicatoria* (EtOH) | 625.0 | 1250 |
| F1 | 2500 | > 2500 |
| F2 (hexane layer) | 1250 | 2500 |
| F3 | > 2500 | > 2500 |
| F4 (tannin-free) | 2500 | > 2500 |
| F5 | > 2500 | > 2500 |
| DMSO% | 12.50 | 12.50 |
| Ciprofloxacin | 1.250 | 1.250 |

[a]Minimum inhibitory concentration

[b]Minimum bactericidal concentration

### *2.5.2. Synergistic antimycobacterial activity on M. tuberculosis using the BACTEC radiometric assay and a colorimetric assay*

The antimycobacterial assays of the extracts against *M. tuberculosis* using the BACTEC radiometric method showed that *K. vesicatoria* inhibited *M. tuberculosis* at a MIC of 50.0 µg/mL. *Pelargonium sidoides* and *E. natalensis* inhibited the bacteria at the 5000 µg/mL and 8.00 µg/mL against *M. tuberculosis* (Table. 2.6.). These results correspond very well with previous MIC values obtained for all of the extracts (section 2.4.4.3.). The sensitive strain of *M. tb* was not susceptible to either F2 (Hexane layer) or F4 (tannin-free extract) obtained from the *K. vesicatoria* tannin clean-up procedure at the highest concentration tested (100 µg/mL). The antituberculosis positive drug, INH inhibited the growth of *M. tuberculosis* at 0.2 µg/mL during all the independent assays.

The combination drug action showed that only C1 and C3 exhibited synergistic antimycobacterial activity. Although the combined MIC of C1 [*E. natalensis* ($CHCl_3$) + *K. vesicatoria* (EtOH) + *P. sidoides* (EtOH) + INH] was reduced eight-fold from 5058.2 to 632.30 µg/mL, the FIC (0.5) indicates that the combination had threshold synergistic activity. This is mainly due to the high ratio of *P. sidoides* (5000 µg/mL equal to 98.8%) present in the combination which increases the MIC of INH (0.20 µg/mL) more than 3000 times over while the presence of the INH actually decreases the MIC of *P. sidoides* eight-fold. The same situation, where the *P. sidoides* extract overwhelms the

other components in the amalgamation, is seen with C2 [*E. natalensis* ($CHCl_3$) + *K. vesicatoria* (EtOH) + *P. sidoides* (EtOH)]. Here the *P. sidoides* extract activity is halved from 5000 to 2529 µg/mL, giving the impression that the activity of the whole drug combination has doubled. However, the extract of *K. vesicatoria* is being subjected to an MIC increase of more than 50 times (from 50.00 to 2529 µg/mL) and *E. natalensis* an increase of more than 300 (from 8.00 to 2529 µg/mL) which is slightly reflected by the FIC (1.5), indicating an additive effect of the combination. The best synergistic result with an FIC of 0.25 was seen for C3 [*K. vesicatoria* (EtOH) + INH] where the combined MIC is also reduced eight-fold, from 50.20 to 6.275 µg/mL. The concentration indicates that the activity of *K. vesicatoria* is increased eight-fold but the activity of INH is decreased 30 times over from 0.20 µg/mL to 6.275 µg/mL. The fact remains that the activity of *K. vesicatoria* was increased eight-fold. This implies that if a patient were to take the same amount of INH prescribed for the treatment of TB but combined this regime with the extract of *K. vesicatoria* (at an eighth of its concentration that would have had to be taken alone) other possible benefits such as decreased toxicity, increased bioavailability and/or immunostimulation could subsist. Testing the cytotoxicity, intracellular antimycobacterial activity and immunomodulation of this combination is the next step to shed some light on these possibilities.

**Table. 2.6. MIC values of extracts, fraction and synergistic combinations against *M. tuberculosis***

| Tested samples | MIC (µg/mL) | FIC[a] | $\Delta GI_{4-3}$ ± SD[b] |
|---|---|---|---|
| V2[c] | - | nd | 22.0 ± 2.94 |
| INH (0.2 µg/mL) | - | nd | -2.50 ± 2.12 |
| *K. vesicatoria* EtOH | 50.00 | nd | -6.00 ± 0.58 |
| F4 (*K. vesicatoria* tannin-free extract) | >100.0 | nd | 275.5 ± 45.3 |
| F2 (Hexane layer) | >100.0 | nd | 288.5 ± 65.1 |
| *E. natalensis* $CHCl_3$ | 8.000 | nd | -3.50 ± 0.88 |
| *P. sidoides* EtOH | 5000 | nd | 20.00 ± 1.85 |
| C1 | 1.000/6.250/625.0/0.025[d] | 0.5 | -5.50 ± 3.53 |
| C2 | 4.000/25.00/2500[e] | 1.5 | 17.00 ± 5.65 |
| C3 | 6.250/0.025[f] | 0.25 | -1.5 ± 0.707 |

[a]Fractional inhibitory concentration

[b]ΔGI value (mean ± standard deviation)

[c]$10^{-2}$ inoculum control

[d]Eight-fold reduction of respective MIC values for [*E. natalensis* ($CHCl_3$) + *K. vesicatoria* (EtOH) + *P. sidoides* (EtOH) + INH]

[e]Two-fold reduction of respective MIC values for [*E. natalensis* ($CHCl_3$) + *K. vesicatoria* (EtOH) + *P. sidoides* (EtOH)]

[f]Eight-fold reduction of respective MIC values for [*K. vesicatoria* (EtOH) + INH]

**Table. 2.7. Antimycobacterial activity of *K. vesicatoria* (EtOH) against two clinical drug-resistant strains of *M. tuberculosis***

| Tested samples | 4388[a] | | 5479[b] | |
|---|---|---|---|---|
| | MIC (µg/mL) | $\Delta GI_{4-3}$ ± SD | MIC (µg/mL) | $\Delta GI_{3-2}$ ± SD |
| V2 | - | 32.5 ± 4.95 | - | 34.5 ± 0.71 |
| *K. vesicatoria* EtOH | 50.00 | 2.0 ± 4.24 | 50.00 | 0.33 ± 0.57 |

[a]Two drug resistant strain (INH and EMB)

[b]Four drug resistant strain (INH, EMB, STR, RIF)

*Knowltonia vesicatoria* inhibited both drug resistant strains of *M. tuberculosis* at a MIC of 50.0 µg/mL (Table. 2.7.). This indicates a mechanism of action different to INH, EMB, STR and RIF. These drugs target cell wall synthesis (INH, EMB), inhibits gene transcription (INH, RIF) and inhibits protein synthesis (STR; Zhang, 2005).

**Table. 2.8. Antimycobacterial activity: Comparison of the BACTEC radiometric assay to the INT colorimetric assay**

| Sample | BACTEC MIC (µg/mL) | INT MIC (µg/mL) |
|---|---|---|
| INH | 0.20 | 0.20 |
| *K. vesicatoria* (EtOH) | 50.0 | 25.0 |
| *E. natalensis* ($CHCl_3$) | 8.00 | 16.0 |
| C3 | 6.28 | 12.5 |

As shown in Table 2.8., the INT results were reproducible for INH (0.2 µg/mL) compared with that obtained by the BACTEC radiometric method. It is noteworthy that even though MIC determinations for INH was not affected by the culture conditions, the MIC values of the chloroform extract of *E. natalensis* and that of C3 doubled, from 8.00 to 16.0 µg/mL and 6.28 to 12.5 µg/mL, respectively. The low metabolic activity of mycobacteria during their very early growth and the slowing metabolic activity of mycobacteria during the stationary phase of growth may limit the entrance of these samples into bacilli, resulting in higher MICs. Another factor can also include the effect of catalase present in the OADC enrichment of the 7H9 broth which utilises hydrogen peroxide to oxidise toxins including phenols (Scandalios *et al*., 1997). The extract of *K. vesicatoria* was the only sample that showed a decrease in MIC value, from 50.0 to 25.0 µg/mL. This effect can also be due to many factors including increased entrance of these samples into bacilli due to surfactant-like molecules such as saponins present in the extract. It is interesting to note that the activity of C3 halved, even though its constituents (INH and *K. vesicatoria*) exhibited the same or increased activity. Results were obtained after 5-7 days incubation, with a length of evaluation time similar to that of the BACTEC method. The direct colorimetric microplate dilution assay employing INT as the growth indicating agent is a rapid, simple and inexpensive alternative to the BACTEC radiometric assay. The MABA or Alamar Blue assay which is frequently employed for antimycobacterial microplate assays, is a proprietary reagent that requires stabilizing agents and

preferably a microplate spectrophotometer to accurately evaluate colour development. Tetrazolium salt assays, such as MTT and TTC have been reported to require lysing buffer such as sodium dodecyl sulphate (SDS) for colour development which could interfere with mycobacterium viability (Banfi *et al*., 2003). During the present study, colour development of the INT still occurred without the addition of a lysing buffer. Direct visual observation of colour change from yellow to pink was adequate: results were always assessed without instrumentation for photometric or fluorimetric reading. The use of a concentration of $10^5$ mycobacteria/mL as inoculum was sufficient to ensure a positive growth response after six day incubation. Although it should be mentioned that the colour development was not present at the same intensity with all the independent repeats. A more reliable method such as the resazurin microdilution assay, MRA, (Banfi *et al*., 2003) which also does not need stabilizing agents or a microplate spectrophotometer can prove to be a better option for a direct colorimetric microplate assay.

## 2.6. Conclusion

Fractions obtained from the tannin clean-up of the *K. vesicatoria* ethanol extract, exhibited no significant antimycobacterial activity when tested against two mycobacteria, *M. smegmatis* and *M. tuberculosis.* The highest activity was for the hexane fraction (F2, containing non-polar compounds) which had a MIC of 1250 µg/mL against the non-pathogenic mycobacteria. The tannin-free fraction (F4), exhibited poor antimycobacterial activity with a MIC of 2500 µg/mL against *M. smegmatis* and a MIC of above 100 µg/mL against *M. tb*. The activity of the whole *Knowltonia* extract remained superior with a MIC of 625 µg/mL against *M. smegmatis* and a MIC of 50.0 µg/mL against one sensitive strain and two drug-resistant strains of *M. tb*. The most likely scenario is that non-polar components synergistically interact with polar components to exert the eventual inhibitory activity seen for the whole extract.

According to Williamson (2002) the method for determining the FIC as described by (Bapela *et al*., 2006; Berenbaum 1978; De Logu *et al*., 2002) would be valid under conditions such as the mechanism of action being similar for all agents, and a linearity of response but that these conditions are unlikely to apply to complex herbal mixtures. The combination of *K. vesicatoria* with INH (C3) resulted in pronounced synergistic activity. Together with Williamson's view, on similar mechanisms of action resulting in true synergy, and the inhibitory activity of the *K. vesicatoria* extract against two INH resistant strains of *M. tb,* a mechanism of action for the extract being very different to INH is plausible. Testing the cytotoxicity, intracellular antimycobacterial activity and immunomodulation of this combination is the next step to shed some light on other synergistic possibilities.

The direct colorimetric microplate dilution assay employing INT as the growth indicating agent, is a rapid, simple, inexpensive, low technology procedure suitable for susceptibility testing or for screening new antimycobacterial compounds against *M. tuberculosis*. Further standardisation of the test is necessary and should include susceptibility testing of all the current first-line antitubercular drugs.

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# CHAPTER 3
# IDENTIFICATION and ISOLATION of BIOACTIVE COMPOUNDS from *Knowltonia vesicatoria*

## 3.1. Introduction

Medicinal plants play an important role in the discovery of potential innovative leads in drug development and in the development of high quality herbal drugs with proven efficacy and development of new research strategies. Detection of biologically active natural products plays a key role in the phytochemical investigation of crude plant extracts. Selection of plants by ethnobotanical and chemotaxonomic criteria offers a good probability of finding candidates which contain compounds active against mycobacteria (Cantrell *et al*., 1998; Lall and Meyer, 1999). Very few compounds have been isolated from *K. vesicatoria*. Compounds known to be present in this species include protoanemonin which readily dimerizes to anemonin. The ethanol extract of *K. vesicatoria* aerial parts exhibited antimycobacterial activity against *M. smegmatis* with a MIC of 625 µg/mL (Labuschagné, 2008). The extract also revealed high activity against a sensitive and two resistant strains of *M. tb* with a MIC of 50 µg/mL against all three strains (section 2.5.1. and 2.5.2.). The objective in this chapter is to isolate, purify and characterize secondary metabolites with antimycobacterial activity from the aerial parts of *K*. *vesicatoria*.

## 3.2. Materials and methods

### *3.2.1. Isolation of pure compounds from K. vesicatoria*

Considering the significant inhibitory activity shown by the ethanol extract of *K*. *vesicatoria* against *M*. *smegmatis* and *M*. *tuberculosis*, this plant species was selected for the identification of bioactive principles. As described previously (section 2.4.1.), air-dried powdered aerial parts of *K. vesicatoria* was extracted with ethanol (EtOH) yielding 148.40 g dried extract. Approximately 28g of the extract was subjected to silica column chromatography (CC, size 10 x 23 cm; Fig. 3.1.) using hexane:ethylacetate (EtOAc) mixtures of increasing polarity (0 to 100%) followed by methanol (MeOH). In total, 26 sub-fractions were collected (500 mL). Similar fractions, according to the thin layer chromatographic (TLC) profile, were combined into 9 main fractions (F1-9; Fig. 3.2.). The fractions were assayed on a thin layer chromatogram (TLC silica gel 60 $F_{254}$) using Dichloromethane and MeOH (9:1) as eluent and analysed under UV spectrum. These 9 primary fractions were concentrated to dryness under reduced pressure and subjected to a TLC bioautographic antibacterial assay using *M. smegmatis*. All the fractions contained a non-polar

constituent with slight activity against *M. smegmatis*, F1-3 exhibited the most obvious inhibition (Fig. 3.3.).

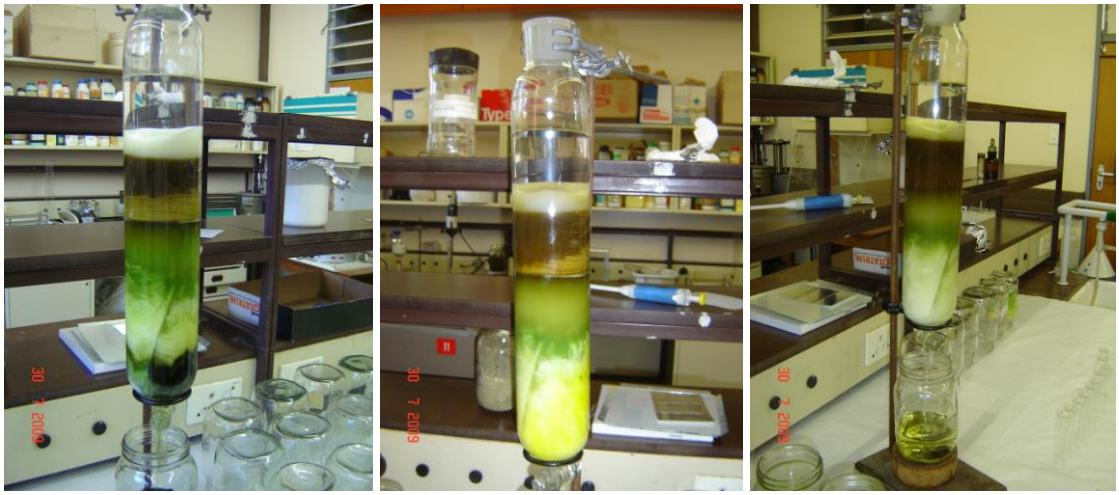


**Fig. 3.1. Silica column chromatography of the EtOH extract from *K. vesicatoria* aerial parts**

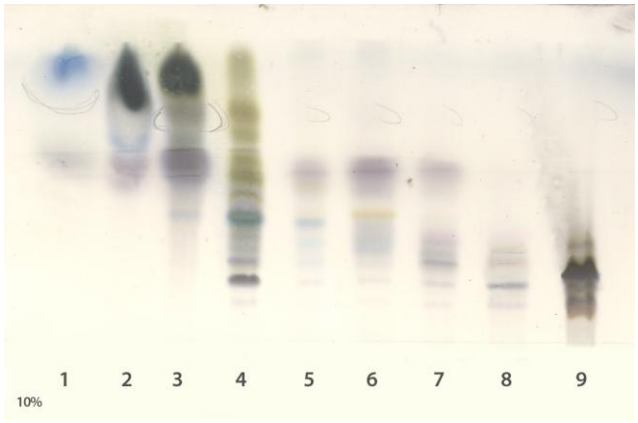



**Fig. 3.2. Combined fractions (F1-F9) from the initial *K. vesicatoria* (EtOH) silica column. Solvent system: Dichloromethane:MeOH (9:1)**

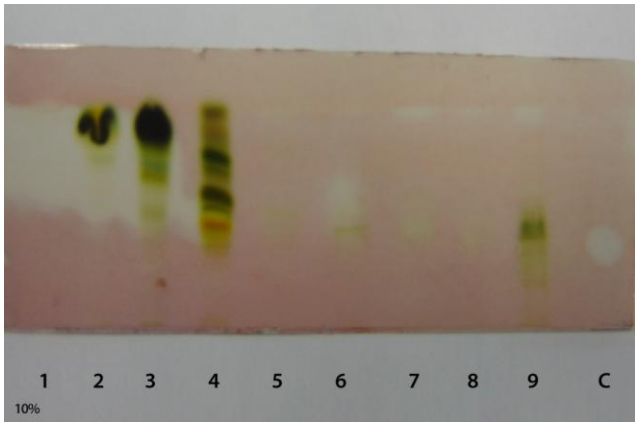
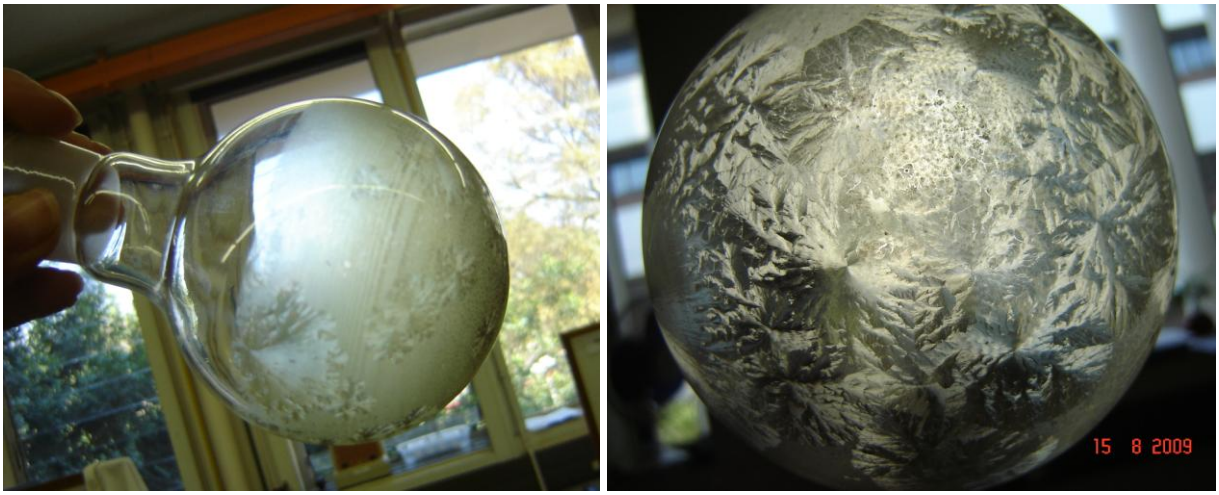



**Fig. 3.3. Bioautographic TLC assay of F1-F9 against *M. smegmatis*. White spots indicate zones of mycobacteria inhibition. Ciprofloxacin (1mg/mL) used as a positive control. Solvent system: Dichloromethane:MeOH (9:1)**

Fraction 3 (smelled like mustard/pepper) contained a prominent active compound (grey band circled with pencil; Fig. 3.2.) not present in any of the other fractions with inhibitory activity (Fig. 3.3.) and was chosen for further purification. This fraction (F3, 404.0 mg) was subjected to silica gel CC (size 3 x 25cm) gradient elution from hexane to ethylacetate (0 to 60%) which yielded 74 sub-fractions (20 mL). Again the sub-fractions were pooled together according to TLC, yielding 12 primary fractions (F3.1-12). Fraction 3.8 (59.30 mg) formed crystals after drying (Fig. 3.4.a.) and was rechromatographed (Fig. 3.4.b.) with a silica gel column (CC, size 2.5 x 27cm) using a hexane:ethylacetate mixture (9:1) to eventually yield the sterol Stigmasta-5,23-dien-3-ol (30.70 mg, Fig. 3.7.).

**(a)**




**(b)**

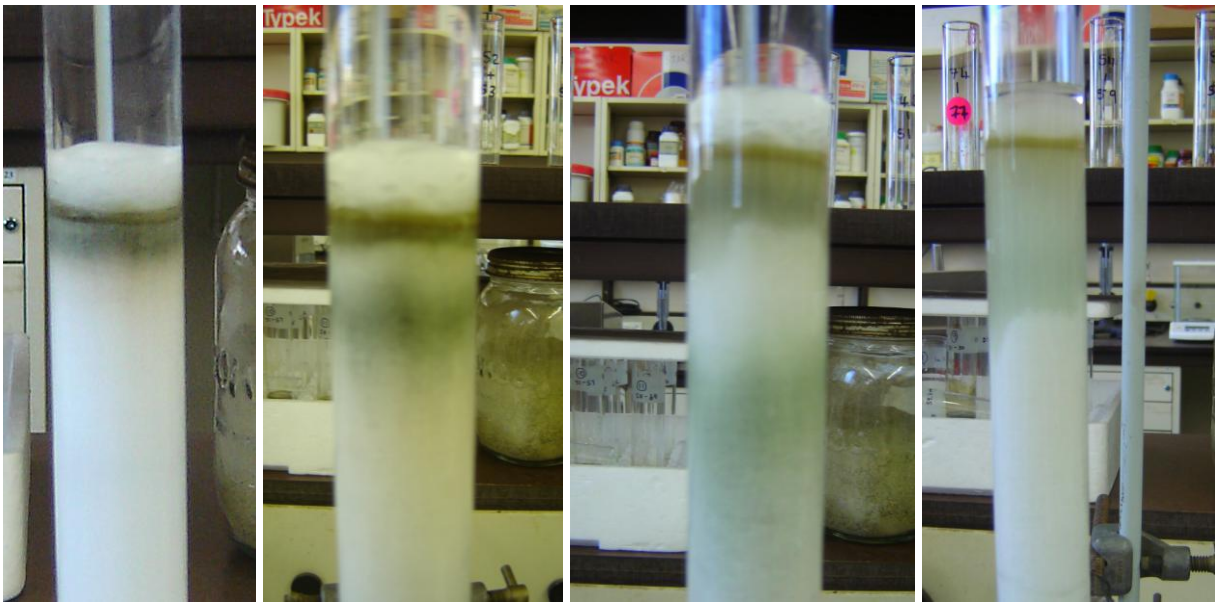
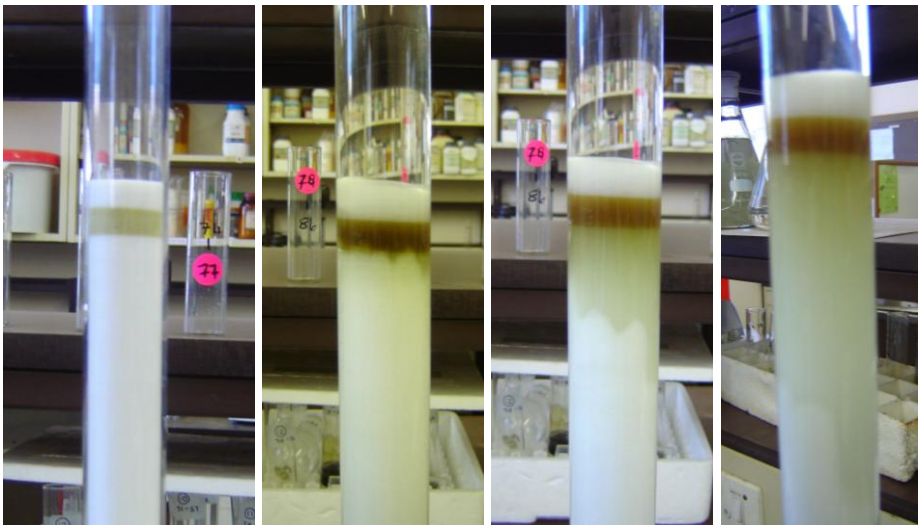



**Fig. 3.4. (a) Clear crystals of F3.8 after drying (b) Silica column chromatography of F3.8**

Another fraction from the first column, F8 (378.9 mg), contained very few constituents with one main compound (dark blue band; Fig. 3.2.). Eventhough no significant inhibitory activity was evident for this fraction, preliminary spectroscopic ($^{1}H$ and $^{13}C$ NMR) data of F8 indicated that the major compound consisted of complex ring structures, indicative of a derivative of anemonin.

Based on the amount of fraction available, the simplicity of constituents and the possibility of a novel compound, this fraction was chosen to be subjected to silica gel CC (hexane-ethylacetate, 30 to 100%). Fifty sub-fractions were obtained and pooled together as two main fractions (F8.1 and F8.2). Fraction 8.2 was further subjected to sephadex CC (3.5 x 23cm) eluted with distilled EtOH. The sephadex column resulted in 78 sub-fractions that were pooled together to yield 5 main fractions (F8.2.I-V; Fig. 3.5.b.). Fraction F8.2.III resulted in the 3:2 isomer mixture of *5-(hydroxymethyl)furan-2(5*H*)-one* and its enantiomer *5-(hydroxymethyl)dihydrofuran-2(3*H*)-one* (123.3 mg; Fig. 3.6.) which was originally thought to be a single derivative of anemonin. The structural identity of the mixture was elucidated by their physical (mp. $[\alpha]_D$) and spectroscopic ($^1$H and $^{13}$C NMR) data and was also subjected to 2D COSY, HMQC, HMBC and NOESY spectra.

**(a)**



**(b)**

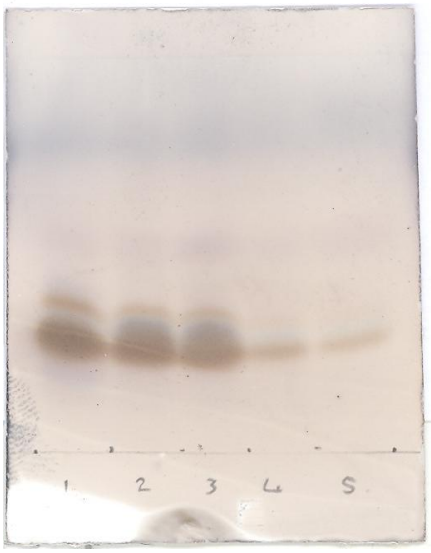



**Fig. 3.5. (a) Silica column chromatography of F8 (b) Detection: vanillin in $H_2SO_4$**

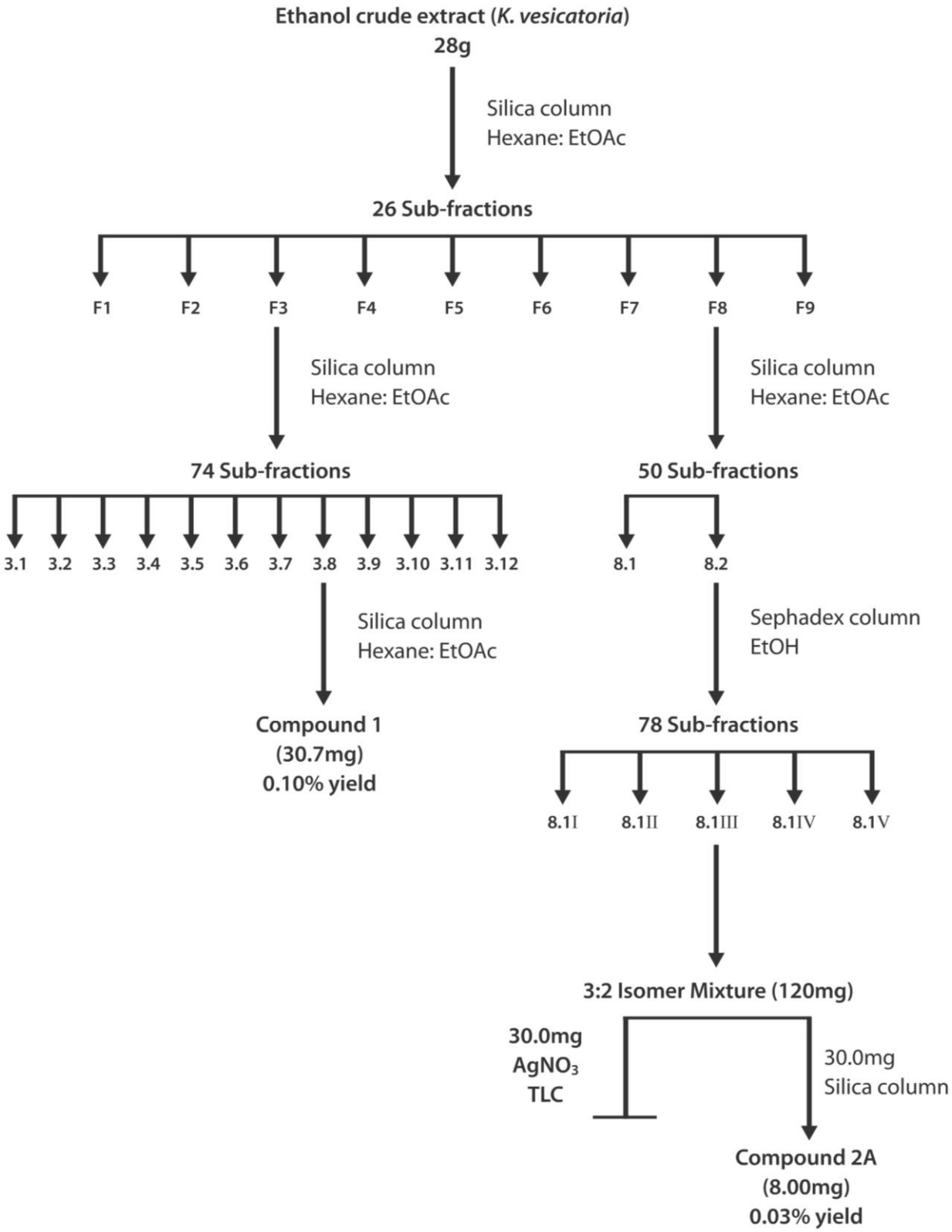



**Fig. 3.6. Schematic representation of the purification steps for the isolation of the compounds from the ethanol extract of *K. vesicatoria***

Separation of the isomers was later attempted, first with silver nitrate ($AgNO_3$) preparative TLC, which was unsuccessful. Of the remaining isomer mixture, 30mg was subjected to column chromatography (CC, 3 x 30cm) using 150 g of inactivated silica (1mL $dH_20$ for each 10g of silica) eluted with hexane:EtOAc (40 to 60%). This procedure eventually separated a portion of the main isomer (*5-(hydroxymethyl)furan-2(5*H*)-one*) from the mixture, yielding a total of 8mg. Isolation of the minor isomer (*5-(hydroxymethyl)dihydrofuran-2(3*H*)-one*) was unsuccessful, with subsequent column chromatography the compound was absorbed by the column.

### *3.2.2. Identification of the isolated compounds*

**Compound 1: Stigmasta-5,23-dien-3-ol** (Fig. 3.7.) was obtained as clear crystals; $^{1}$H NMR (500 MHz, $CDCl_3$): δ 0.683 (3H, s, C-18), 1.004 (3H, s, C-19), 0.835 (3H, d, *J* = 6.5 Hz, C-21), 0.997 (6H, d, *J* = 7.1 Hz, C-26, C-27), 0.946 (3H, t, *J* = 7.7 Hz, C-29), 4.90 (1H, dd, *J* = 6.4 Hz, *J* = 7.6 Hz, C-23), 3.517 (1H, m, C-3α), 5.35 (1H, m, C-6), 2.27 (1H, m, C-25) (Fig. 3.8.a.). $^{13}$C NMR (125 MHz, $CDCl_3$): δ 140.7 (C-24), 138.3 (C-5), 129.2 (C-6), 121.6 (C-23), 77.00, 71.76 (C-3), 56.82 (C-14), 55.89 (C-17), 51.20 (C-9), 45.80, 42.25 (C13), 40.48 (C-4), 39.73 (C-12), 37.22 (C-1,C-10), 36.47 (C-20), 36.12 (C-25), 33.90 (C22), 31.86 (C-7), 31.61(C-2/C-8), 29.00, 28.23, 25.50, 23.70, 21.93 (C-26), 21.03 (C-27), 19.80 (C-21), 19.37 (C-19), 12.24 (C-29), 12.00 (C-18) (Fig. 3.8.b.).

The compound showed signals of triterpene skeleton in H and C NMR spectra at δ 0.683, 1.004 (s, Me-18,-19), 0.84, 1.00 (X2), 0.95 (Me-21, 26, 27, 29), in addition to two olefinic and a proton adjacent to hydroxyl group at 4.90 (1H, dd, *J* = 6.4 Hz, *J* = 7.6 Hz, C-23), 5.35 (1H, m, C-6), 3.517 (1H, m, C-3α). The C NMR data showed 29 carbons and classified according to DEPT-135 into 6 methyls, 10 methylenes, 9 methines, and 4 quaternary carbons. The above data are typical with those reported in literature (Steiner *et al*., 1977 and Itoh *et al*., 1983) for the same compound.

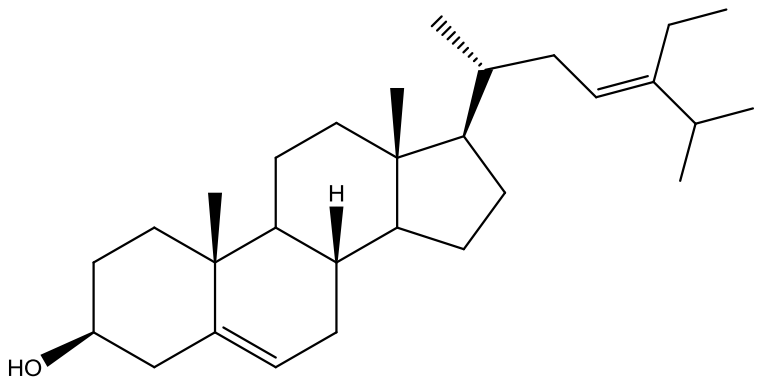
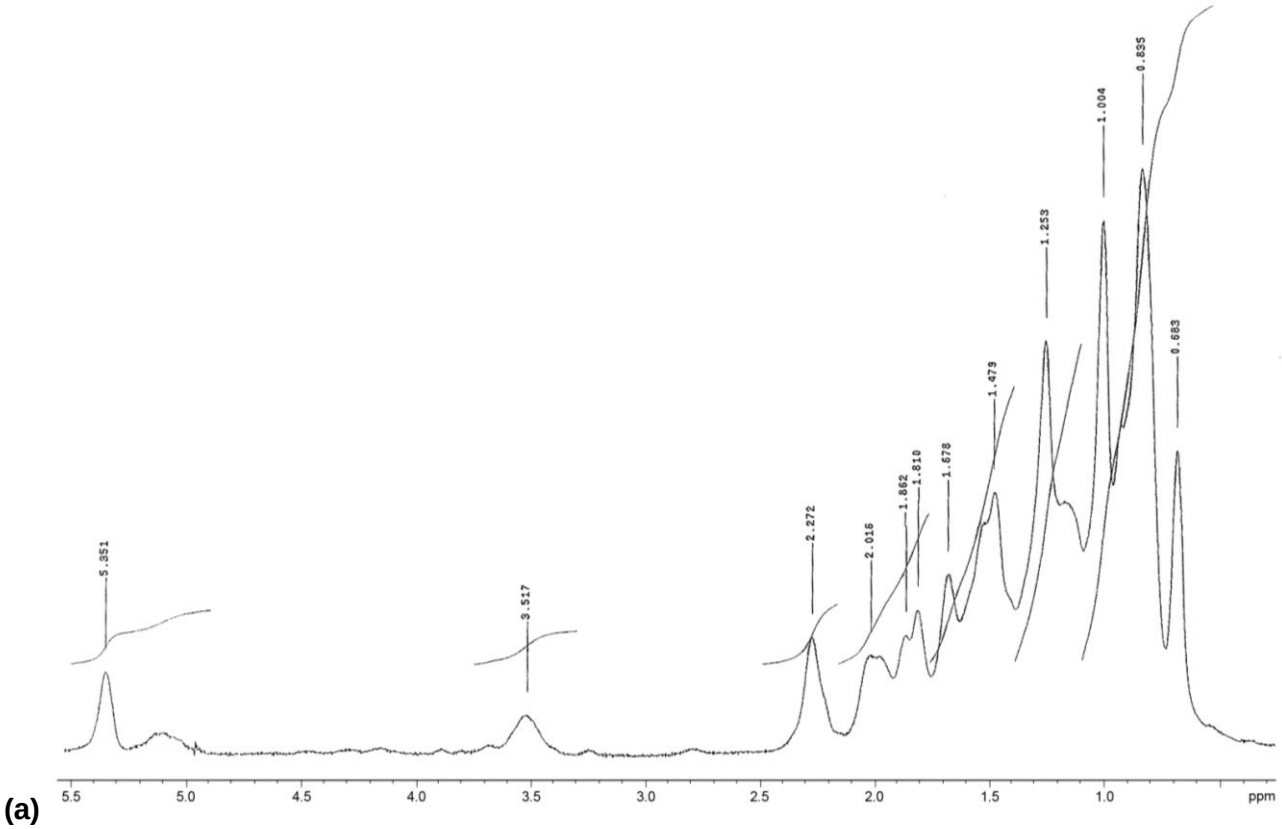
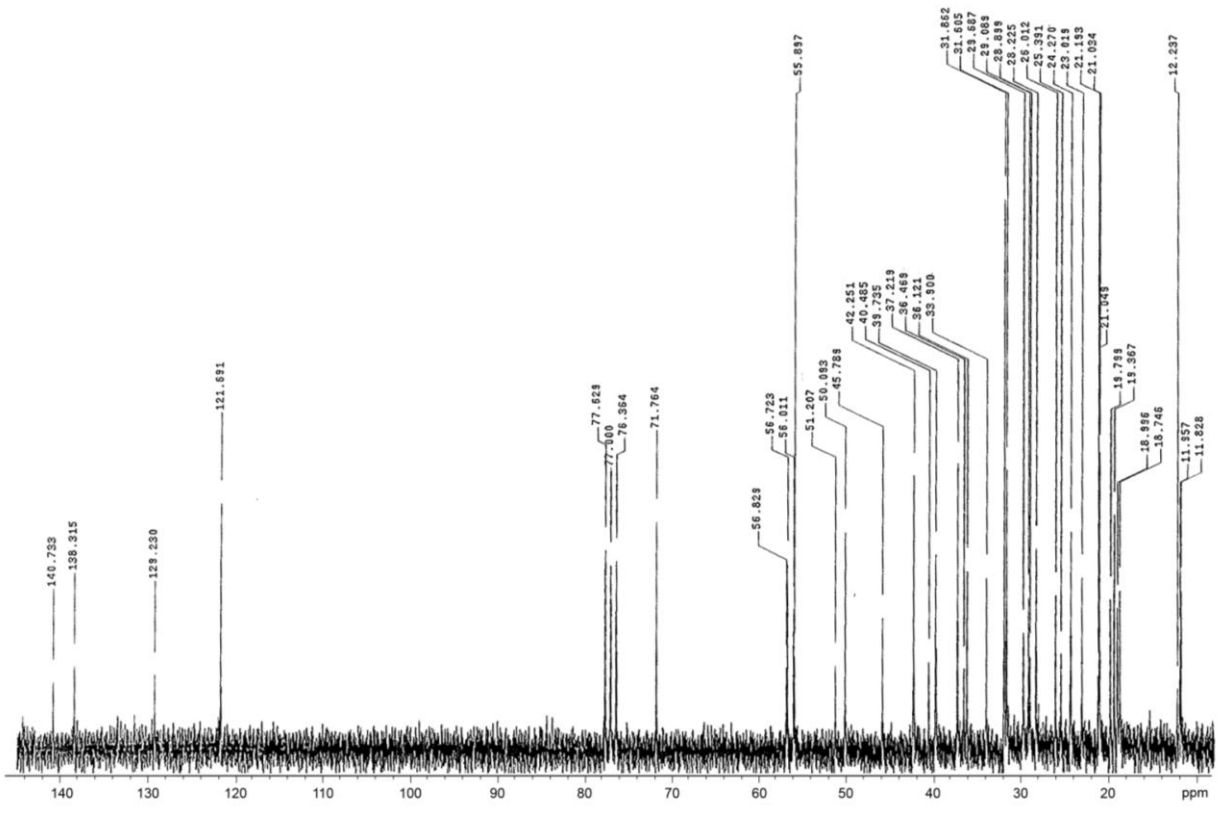



**Fig. 3.7. Structure of Compound 1: Stigmasta-5,23-dien-3-ol**




(a)




(b)

**Fig. 3.8. NMR spectrums of Compound 1 (Stigmasta-5,23-dien-3-ol)**

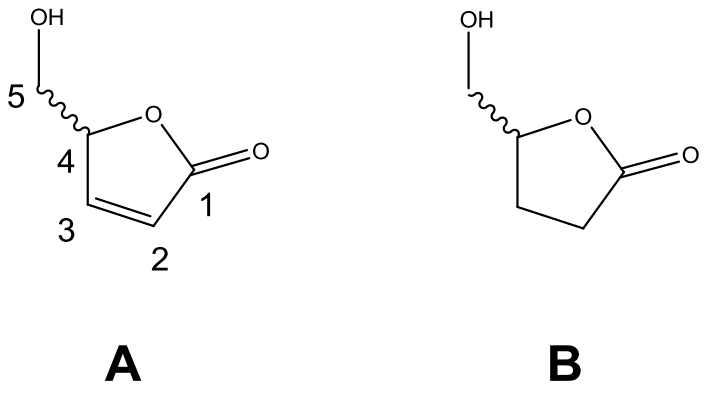
**(a) $^{1}$H NMR spectra**

**(b) $^{13}$C NMR spectra**

**Isomer mixture: *5-(hydroxymethyl)furan-2(5H)-one* and *5-(hydroxymethyl)dihydrofuran-2(3H)-one*** (Fig. 3.9.) was obtained as a yellow oil. This sample is a ≈ 3:2 mixture of compounds **A** and **B**, respectively, as was revealed by the integral of the $^{1}$H NMR spectrum and also by the absence of HMBC and COSY correlations between the signals corresponding to each one of the constituents. The absolute stereochemistry at the C-4 asymmetric centre not determined.

**Compound 2A: *5-(hydroxymethyl)furan-2(5H)-one***

$^{1}$H NMR (500 MHz, $CDCl_3$): δ 7.47 (1H, dd, $J_{3,2}$ = 5.8 Hz, $J_{3,4}$ = 1.6 Hz, H-3), 6.21 (1H, dd, $J_{2,3}$ = 5.8 Hz, $J_{2,4}$ = 2.1 Hz, H-2), 5.15 (1H, dddd, $J_{4,2}$ = 2.1 Hz, $J_{4,3}$ = 1.6 Hz, $J_{4,5A}$ = 3.9 Hz, $J_{4,5B}$ = 5.1 Hz, H-4), 4.00 (1H, dd, $J_{5A,5B}$ = 12.2 Hz, $J_{5A,4}$ = 3.9 Hz, $H_A$-5), 3.79 (1H, dd, $J_{5B,5A}$ = 12.2 Hz, $J_{5B,4}$ = 5.1 Hz, $H_B$-5). $^{13}$C NMR (125 MHz, $CDCl_3$): δ 172.8 (qC, C-1), 153.2 (CH, C-3), 123.1 (CH, C-2), 83.8 (CH, C-4), 62.5 ($CH_2$, C-5).




**A** **B**

**Fig. 3.9. Structures for the isolated enantiomer mixture of *5-(hydroxymethyl)furan-2(5H)-one* (A) and *5-(hydroxymethyl)dihydrofuran-2(3H)-one* (B)**

**Compound 2B: *5-(hydroxymethyl)dihydrofuran-2(3H)-one***

$^{1}$H NMR (500 MHz, $CDCl_3$): δ 4.64 [1H, (*0.8 H in the spectrum*), m (dddd, Σ*J* = 22.2 Hz), H-4), 3.91 (1H, dd, $J_{5A,5B}$ = 12.5 Hz, $J_{5A,4}$ = 2.9 Hz, $H_A$-5), 3.66 (1H, dd, $J_{5B,5A}$ = 12.5 Hz, $J_{5B,4}$ = 4.6 Hz, $H_B$-5), 2.63 (1H, ddd, $J_{2A,2B}$ = 17.8 Hz, $J_{2A,3B}$ = 10.0 Hz, $J_{2A,3A}$ = 5.6 Hz, $H_A$-2), 2.55 (1H, m, Σ*J* = 36.2 Hz, $H_B$-2), 2.27 (1H, m, Σ*J* = 35.9 Hz, $H_A$-3), 2.15 (1H, m, Σ*J* = 38.2 Hz, $H_B$-3). $^{13}$C NMR (125 MHz, $CDCl_3$): δ 177.2 (qC, C-1), 80.5 (CH, C-4), 64.2 ($CH_2$, C-5), 28.6 ($CH_2$, C-2), 23.2 ($CH_2$, C-3).

All these assignments were in agreement with the COSY, HSQC and HMBC spectra. -HRESIMS of the mixture **A** + **B**: found 115.0393 $[M+H]^+$ for **A**, calculated for $C_5H_7O_3$ 115.03951; found 117.0548 $[M+H]^+$ for **B**, calculated for $C_5H_9O_3$ 117.05517.

After the successful separation of A, the structural identity of the compound was confirmed with its specific rotation ( $[\alpha]_D$) and spectroscopic ($^1$H NMR) data.

**Compound 2A: *5-(hydroxymethyl)furan-2(5H)-one***

$[\alpha]_D$ -5.7 (*c* 0.16, $CHCl_2$); $^1$H NMR (500 MHz, $CDCl_3$): δ 7.47 (1H, dd, $J_{3,2}$ = 5.8 Hz, $J_{3,4}$ = 1.6 Hz, H-3), 6.21 (1H, dd, $J_{2,3}$ = 5.8 Hz, $J_{2,4}$ = 2.1 Hz, H-2), 5.15 (1H, dddd, $J_{4,2}$ = 2.1 Hz, $J_{4,3}$ = 1.6 Hz, $J_{4,5A}$ = 3.9 Hz, $J_{4,5B}$ = 5.1 Hz, H-4), 4.03 (1H, dd, $J_{5A,5B}$ = 12.2 Hz, $J_{5A,4}$ = 3.9 Hz, $H_A$-5), 3.79 (1H, dd, $J_{5B,5A}$ = 12.2 Hz, $J_{5B,4}$ = 5.1 Hz, $H_B$-5) (Fig. 3.10.).

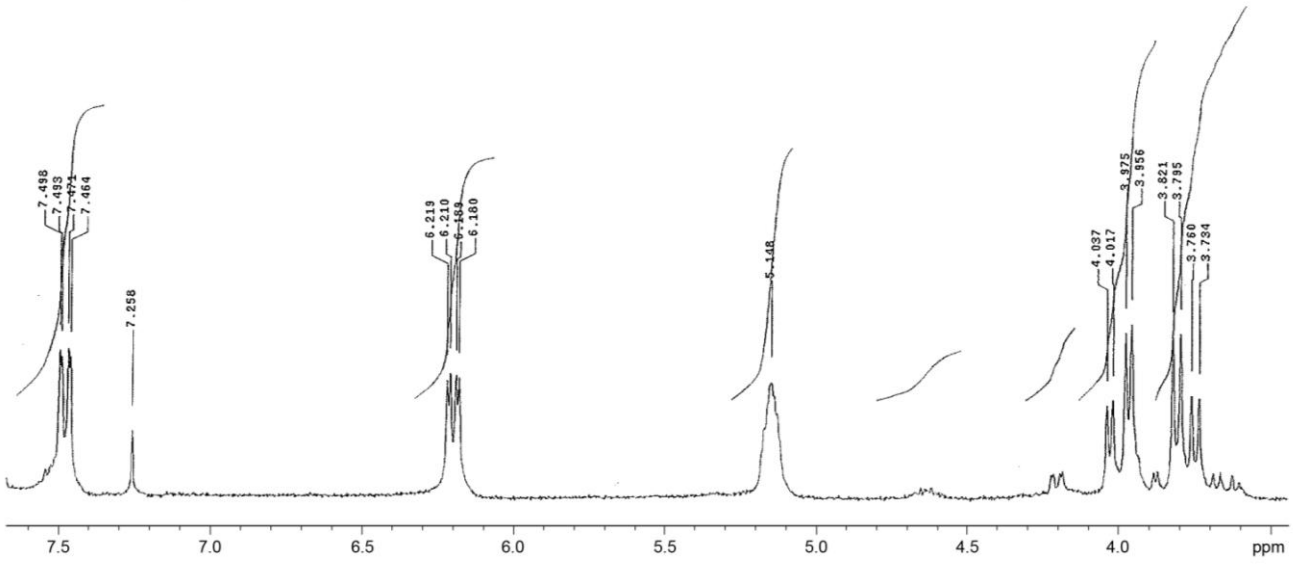



**Fig. 3. 10. $^1$H NMR spectra of Compound 2A (*5-(hydroxymethyl)furan-2(5H)-one*)**

### *3.2.3. Antimycobacterial activity of isolated compounds on M. tuberculosis using the rapid radiometric assay and the INT colorimetric assay*

The procedure for the BACTEC radiometric assay was carried out as mentioned in Chapter 2 (section 2.4.4.3.) and the same procedure was carried out for the INT microplate dilution assay as mentioned in section 2.4.4.5.

#### 3.2.3.1. Preparation of Micro-organisms

The drug-susceptible strain of *M. tuberculosis* H37Rv (ATCC 27294) was maintained and prepared as before (section 2.4.4.2.).

#### 3.2.3.2. Preparation of compounds

The compounds were tested in triplicate at four concentrations ranging from 200 to 25.00 µg/mL, the concentration of DMSO never exceded 1% during either assay.

## 3.3. Results and discussion

### *3.3.1. Isolation and identification of active compounds from K. vesicatoria*

Isolation of active compounds from *K. vesicatoria* yielded the phytosterol, stigmasta-5,23-dien-3-ol with a total yield of 0.1% (30.7 mg) from the crude extract (28.00 g) and a lactone *5-(hydroxymethyl)furan-2(5*H*)-one* with a yield of 0.028% (8.00 mg) of the total extract.

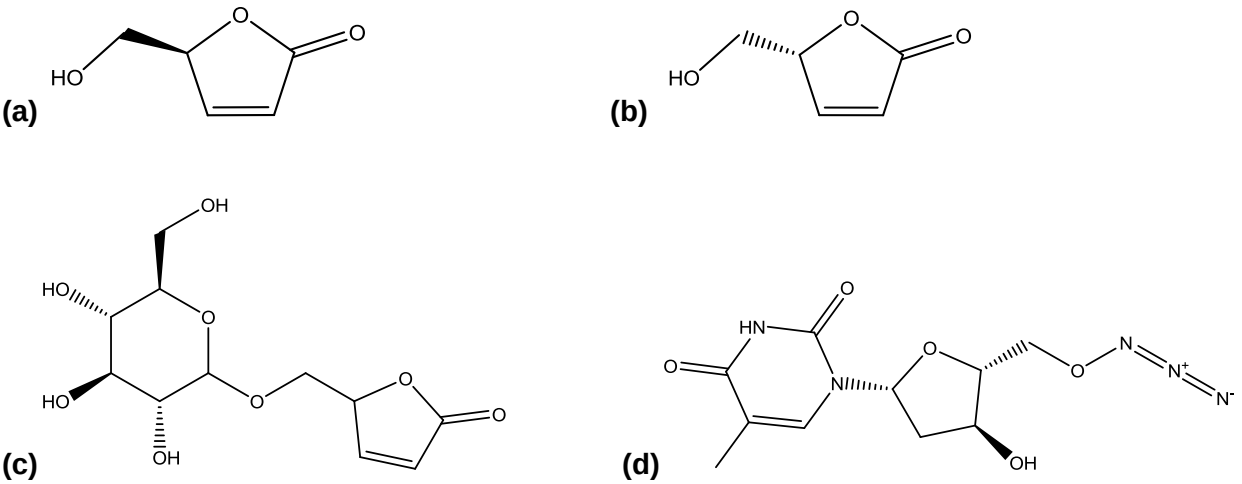



**Fig. 3.11. Chemical structures of**
**(a) (*S*)-5-*(hydroxymethyl)furan-2(5*H*)-one***
**(b) (*R*)-5-*(hydroxymethyl)furan-2(5*H*)-one***
**(c) Ranunculin**
**(d) AZT**

The sterol stigmasta-5,23-dien-3-ol was first isolated in 1977 from the the sponge *Calyx nicaeensis* (Steiner *et al*., 1977). This sterol alcohol falls under the rare $\Delta^{23}$-form of the unsaturated steroids and it is commonly found in olive, oat and grapeseed oil but has never been isolated from *K. vesicatoria*. Additionally, it has been suggested that sterols have anti-inflammatory, antibacterial, antifungal, anti-ulcerative, antioxidant and antitumoral activities (Stuchlík and Žák, 2002; Awad *et al*., 2000). The lactone *5-(hydroxymethyl)furan-2(5*H*)-one* has never been isolated from *K. vesicatoria* or any member of the Ranunculaceae. Since the lactone was obtained as a yellow oily substance, the undefined stereochemistry at the C-4 asymmetric centre is most likely the (*S*)- and not the (*R*)-form (Fig. 3.11.a. and b.), as the latter crystallises and the (*S*)-form is an oil (dnp.chemnetbase.com). The (*S*)-form is a derivative of ranunculin (Fig. 3.11.c.) which is commonly found in the Ranunculaceae family, and ranunculin is the precursor for protoanemonin

(Campbell *et al*., 1979 and Tschesche *et al*., 1981). The (*S*)-form is also an intermediate in the synthesis of the antiretroviral drug azidothymidine (AZT; Fig. 3.11.a; dnp.chemnetbase.com).

### *3.3.2.* Antimycobacterial *activity of compounds against M. tuberculosis*

Table. 3.1. shows the BACTEC and INT MIC values of the two isolated compounds in addition to the values of the isomer mixture (the two lactone enantiomers) against the sensitive strain of *M. tb*. The isomer mixture exhibited no inhibitory activity against *M. tb* at the highest concentration tested in both mycobacterial assays (200.0 µg/mL). Eventhough lactones are known to exhibit significant antibacterial properties, based on the preliminary *M. smegmatis* bioautographic TLC assay of the fractions containing these compounds (section 3.2.2; Fig. 3.3.) it was not surpising that the sterol was considerably more active than the lactone with a MIC of 50.00 µg/mL compared to the lactone MIC of 200.0 µg/mL. It has been reported that phytosterols have anti-inflammatory, antibacterial, antifungal, anti-ulcerative, antioxidant and antitumoral activities (Stuchlík and Žák, 2002; Awad *et al*., 2000). Additionally, Saludes *et al*. (2002) reported the antimycobacterial activity of five phytosterols isolated from *Morinda citrofolia* against *M. tb*. Three of these isolated sterols (Fig. 3.12.) closely resemble the structure of stigmasta-5,23-dien-3-ol (Fig. 3.7.), with the only differences being the position and degree of saturation. Stigmasta-4-en-3-one and stigmasta-4,22-dien-3-one (used in combination) had a MIC lower than 2.00 µg/mL, stigmasterol had an MIC of 32.00 µg/mL. Another phytosterol, saringosterol, isolated from *Lessonia nigrescens*, (a brown algae) exhibited extremely low toxicity compared to its MIC of 0.250 µg/mL (Wächter *et al*., 2000).

**Table. 3.1 Activity of isolated compounds from *K. vesicatoria* against *Mycobacterium tuberculosis***

| Sample | BACTEC assay | | INT colorimetric assay |
|---|---|---|---|
| | MIC (µg/mL) | $\Delta GI_{4-3}$ ± SD[a] | MIC (µg/mL) |
| INH | 0.200 | -2.50 ± 2.12 | 0.200 |
| Compound 1[b] | 50.00 | -9.00 ± 1.41 | 50.00 |
| Compound 2A[c] | nd[d] | nd[d] | 200.0 |
| Isomer mixture | >200.0 | 117.0 ± 8.49 | >200.0 |

[a] ΔGI of the control vial (V2) was 22.0 ± 2.94

[b] Stigmasta-5,23-dien-3-ol

[c] *5-(hydroxymethyl)furan-2(5*H*)-one*

[d] Not determined

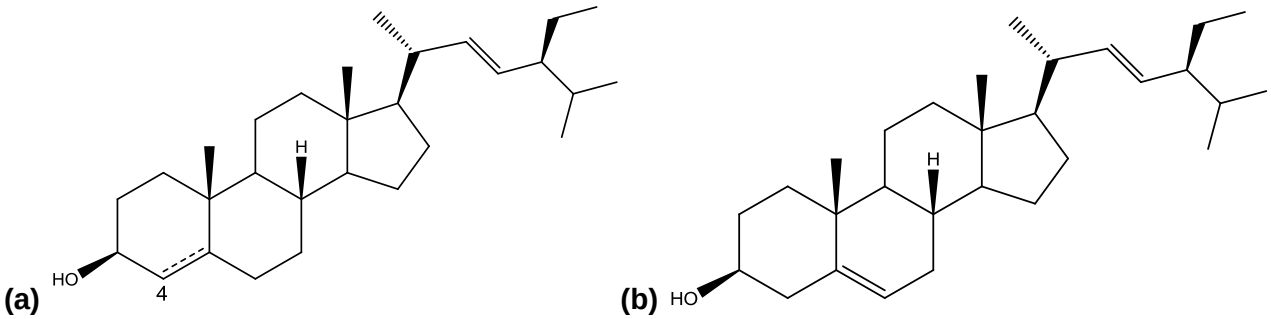



**Fig. 3.12. Structures of antimycobacterial sterols**

**(a) Stigmasta-4-en-3-one, absence of double bond at position 4 and stigmasta-4,22-dien-3-one, presence of double bond at 4**

**(b) Stigmasterol**

## 3.4. Conclusion

The sterol, stigmasta-5,23-dien-3-ol, exhibited the highest antimycobacterial activity of the two isolated compounds with a MIC of 50.00 µg/mL. Taking into consideration the MIC values of the crude EtOH extract of *K. vesicatoria*, which was determined as 50.00 and 25.00 µg/mL via the BACTEC and microdilution assays, respectively, the sterol cannot account for all the antimycobacterial activity of this plant and the hypothesis of synergy within the crude extract still stands. Through bioassay guided fractionation and isolation procedures stigmasta-5,23-dien-3-ol was isolated as a moderately active *in vitro* antimycobacterial component of *K. vesicatoria*. Surprisingly, the lactone *5-(hydroxymethyl)furan-2(*5H*)-one*, exhibited low inhibitory activity of *M. tb*. No previous antimycobacterial activity has been reported for these two compounds.

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# CHAPTER 4
# INHIBITORY and SUBVERSIVE SUBSTRATE ACTIVITY of SELECTED ANTIMYCOBACTRIAL SAMPLES TOWARDS GLUTATHIONE REDUCTASE

## 4.1. Introduction

The flavoprotein disulfide reductase (FDR) family of enzymes initially included lipoamide dehydrogenase (LipDH), glutathione reductase (Gtr), and thioredoxin reductase (Trxr; Williams, 1976). These homodimeric flavoproteins use one molecule of tightly, but noncovalently, bound flavin adenine dinucleotide (FAD) and one redox-active disulfide per polypeptide chain to catalyze the pyridine-nucleotide-dependent (NAD(P)H) reduction of their disulfide-bonded substrates. Subsequent cloning and DNA sequencing of the genes encoding these enzymes and the determination of the three-dimensional structures of the enzymes by X-ray crystallography revealed additional similarity in primary, tertiary, and quaternary structure. On the basis of these criteria, trypanothione reductase (Ttr; discovered in the 1980s) was also added to the family (Williams, 1992), as was the more recently described mycothione reductase (Mtr) from *Mycobacterium tuberculosis* (Patel and Blanchard, 1999).

### *4.1.1. Glutathione Reductase (EC 1.8.1.7, Formerly EC 1.6.4.2) and Mycothione Reductase (EC 1.8.1.15)*

Crystal structures are available for Gtr (Kuriyan *et al*., 1991; Schierbeek *et al*., 1989; Schulz *et al*., 1978), but not for the recently described mycothione reductase. Gtr has monomer folds composed of FAD-binding, pyridine-nucleotide-binding, central, and interface domains. Two identical active sites result from the interaction of two monomers to form the native homodimer (Fig. 4.1.), and each active site contains residues from the first three domains of one monomer and the interface domain of the other, where the His-Glu pair is located. The dimer is the active entity. The active sites of these two enzymes are proposed to be similar with regard to FAD- and pyridine-nucleotide-binding (Argyrou and Blanchard, 2004). The disulfide substrate-binding sites, on the other hand, are different, as each enzyme has evolved to provide specificity for its own substrate. The path of electron transfer is topologically defined: NAD(P)H → FAD → redox-active disulfide → disulfide substrate. (Argyrou and Blanchard, 2004).

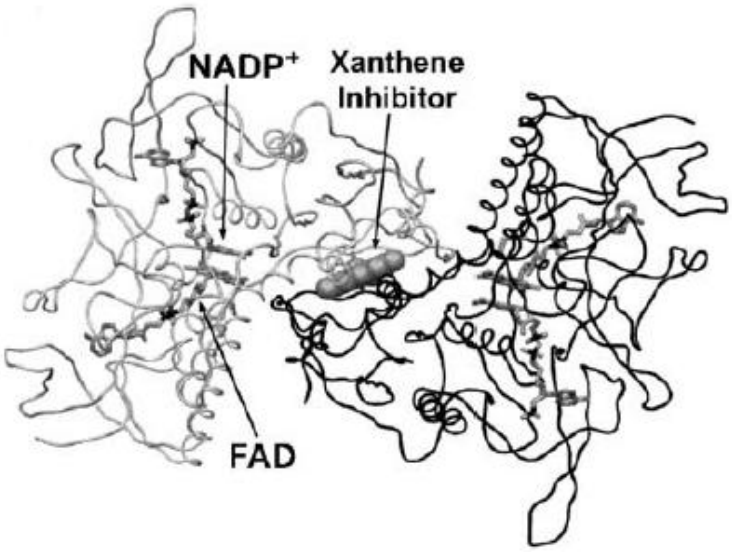


**Fig. 4.1. Structure of homodimeric human Gtr with bound FAD and a xanthene inhibitor (Argyrou and Blanchard, 2004)**

Cells maintain high levels of GSH relative to glutathione disulfide (GSSG; Schirmer and Schulz, 1987) by the action of Gtr, which catalyzes (Williams, 1992) the NADPH-dependent reduction of GSSG to GSH (Eq. 1).

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH \qquad (1)$$

A functional homolog of Gtr, mycothione reductase (Mtr), has been identified from *M. tuberculosis* which catalyzes the NADPH-dependent reduction of mycothione to mycothiol (Eq. 2; Patel and Blanchard, 1999). The specificity of the recombinant enzyme for mycothione is 17- and 1600-fold higher than for des-myo-inositol mycothione (Patel and Blanchard, 1998) and DTNB, respectively, and no activity was detected with GSSG. The specificity for NADPH is 13-fold higher than for NADH. Substituted benzoquinones and naphthoquinones can also be reduced by the enzyme (Patel and Blanchard, 1999). The enzyme has been further characterized by using pH-rate profiles and transient-state kinetics (Patel and Blanchard, 2001).

$$NADPH + H^+ + MSSM \rightarrow NADP^+ + 2MSH \qquad (2)$$

### *4.1.2. Glutathiol and mycothiol*

Reduced glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH; Fig. 4.1.) is the major low-molecular-mass thiol present in nearly all eukaryotes and in many bacteria. It is a cofactor in many processes which protect cells against oxygen toxicity and reactive electrophiles (Newton and Fahey, 2002). By non-enzymatically reacting with reactive oxygen species, it serves as a general buffer against these injurious species. It also serves as a general reductant of aberrant disulfide bonds that form

between proteins and between small thiol-containing molecules. GSH is the substrate for glutathione S-transferases which detoxify a variety of injurious electrophiles, and glutathione peroxidases (Ursini, 1995), which reduce hydrogen peroxide to water. By reducing glutaredoxin, which together with reduced thioredoxin serves as a source of reducing equivalents for ribonucleotide reductase, GSH is also important in deoxyribonucleotide biosynthesis (Holmgren, 1989). GSH is oxidised to the symmetrical glutathione disulfide (GSSG; Fig. 4.2.) by the NADPH-dependent glutathione disulfide reductase (Gtr) which helps to maintain an intracellular reducing environment by reducing GSSG back to GSH (Fig. 4.4.). GSH was thought to be ubiquitous and indispensable in all living cells until it was found that *Escherichia coli* mutants defective in GSH are viable (Apontoweil and Berends, 1975) and that many gram-positive bacteria do not produce GSH (Newton and Fahey, 2002). The GSH-lacking bacteria included many strict aerobes and this prompted a search for other thiols which might play a parallel role to GSH. Methodology for the analysis of known thiols was developed and used in surveys of bacteria lacking GSH (Newton and Fahey, 1995).

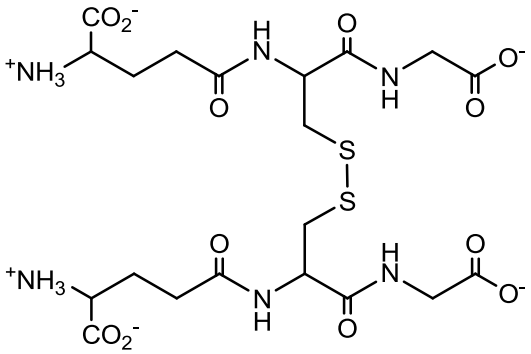

**Fig. 4.2. Chemical structure of Glutathione (GSSG)**

A variety of thiols of unknown structure were detected, including one designated U17 which was found in streptomycetes and other actinomycetes such as *Mycobacterium tuberculosis* and *Streptomyces coelicolor* (Newton and Fahey, 2002). The latter compound proved to be mycothiol (MSH) (Fig. 4.2.) and recent work has indicated that in many gram-positive bacteria mycothiol plays a role similar to that of GSH in GSH-producing organisms (Newton and Fahey, 2002; Rawat and Av-Gay, 2007). A structurally unique low-molecular-weight thiol has been identified as the most abundant thiol in the actinomycetes, which includes the mycobacteria and the streptomycetes. This compound, 1-D-*myo*-inositol-2-(*N*-acetyl-L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside, is a conjugate of *N*-acetylcysteine (AcCys) and can also be written as AcCys-GlcN-Ins (Bzymek *et al*., 2007). This thiol has been given the trivial names mycothione (MSSM; Fig. 4.3.) and mycothiol (MSH) for the oxidized and reduced forms, respectively (Spies and Steenkamp, 1994). MSH is the dominant thiol found in *Mycobacterium tuberculosis* which lacks glutathione, but instead maintains millimolar concentrations of this structurally distinct low molecular weight thiol (Mahapatra *et al*., 2007). Mycothiol is maintained in a reduced state by Mtr,

utilizing reducing equivalents from NADPH (Patel and Blanchard, 1999 and 2001). Like GSH, MSH is believed to play a key role in the inactivation of potentially damaging radicals and reactive oxygen species and is oxidised to the symmetrical mycothiol disulfide (MSSM), in the process. NADPH-dependent mycothiol disulfide reductase (Mtr) helps to maintain an intracellular reducing environment by reducing MSSM back to MSH (Stewart *et al*., 2008; Fig. 4.4.).

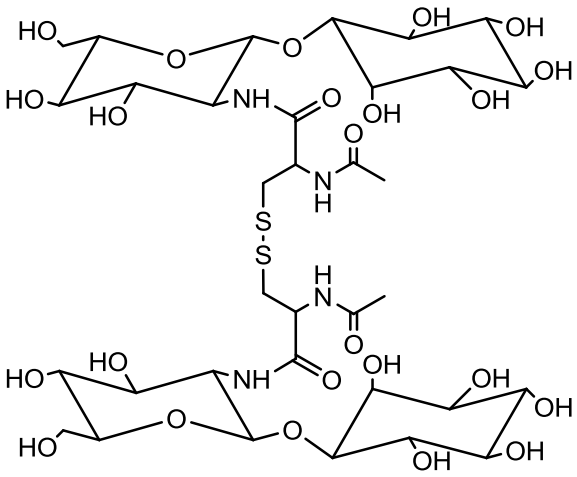


**Fig. 4.3. Chemical structure of Mycothione (MSSM)**

Mycothiol is also a substrate for mycothiol-dependent formaldehyde dehydrogenase (Newton and Fahey, 2002), which, in addition to detoxifying formaldehyde, also has S-nitrosomycothiol reductase activity (Vogt *et al*., 2003) and thus contributes to combating nitrosative stress. Finally, mycothiol detoxifies electrophiles by forming mycothiol S-conjugates. These are subsequently hydrolyzed by an amidase (Newton and Fahey, 2002) to generate N-acetyl-cysteinyl S-conjugates, which are excreted, and the mycothiol precursor, glucosamine-myoinositol, which re-enters the mycothiol biosynthetic pathway.

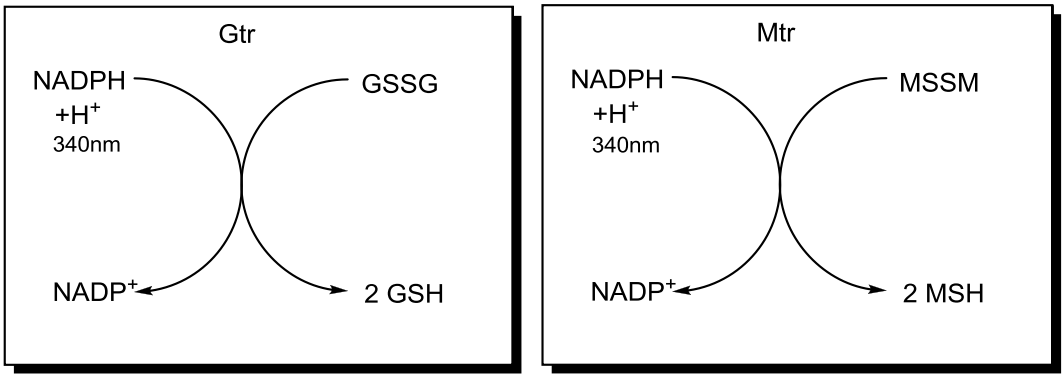


**Fig. 4.4. Schematic representations of the glutathione disulfide reductase (Gtr) and mycothiol disulfide or mycothione reductase (Mtr) pathways (Stewart *et al*., 2008)**

MSH has antioxidant activity as well as the ability to detoxify a variety of toxic compounds. Because of these activities, MSH is a candidate for protecting *M*. *tuberculosis* from inactivation by the immune responses of the mammalian host during infections as well as for resisting antituberculous drugs. Studies have demonstrated that chemically or genetically altered mycobacterial strains that are deficient in MSH production become hypersensitive to most currently used antitubercular antibiotics (Rawat *et al*., 2002; Rawat *et al*, 2004), that exposure to these antibiotics results in upregulation of MSH biosynthesis genes in *Mycobacterium tuberculosis*, BCG (Hayward *et al*., 2004), and that MSH is indeed essential for viability of *M. tuberculosis* (Buchmeier and Fahey, 2006; Bzymek *et al*., 2007; Sareen *et al*., 2003; Sassetti *et al*., 2003). The above observations suggest that substances that can interfere with MSH-assisted detoxification may have therapeutic potential. Mtr enzyme studies are severely restricted by the scarcity of MSH and MSSM, which are difficult to prepare in sufficient quantity. Small quantities of MSH are routinely obtained by whole cell synthesis in typical yields of 1 mg per litre of *M. smegmatis* cell culture, while chemical syntheses are elaborately complex (Stewart *et al*., 2008). In order to define candidates for the selective mechanism of the inhibitory or subversive substrate activity against Mtr, selected antitmycobacterial extracts and isolated compounds were screened against glutathione reductase as a surrogate for Mtr.

## 4.2. Materials and methods

### *4.2.1. Glutathione disulfide reductase assay*

To provide insight into the possible mode of action of antimycobacterial extracts and isolated compounds, and the ability of Gtr and Mtr to turnover antimycobacterial compounds such as naphthoquinones as subversive substrates, the inhibitory and subversive substrate properties of extracts from *K. vesicatoria* (EtOH), *E. natalensis* ($CHCl_3$) and *P. sidoides* (EtOH) were tested against Gtr. The two pure compounds isolated from *K. vesicatoria*, Compound 1 (stigmasta-5,23-dien-3-ol) and and Compound 2A (*5-(hydroxymethyl)furan-2(5*H*)-one*) were also included. Since naphthoquinones are known to operate as subversive substrates with Gtr (Biot *et al*., 2004., Salmon-Chemin *et al*., 2001), it was decided to include a previously isolated naphthoquinone from *E. natalensis*, 7-MJ in this study in order to compare with the compounds isolated from *K. vesicatoria* and the crude *E. natalensis* extract. The naphthoquinone, 7-MJ, was kindly donated by Prof. Lall.

#### 4.2.1.1. Absorption values

To determine the highest possible concentration of crude plant extract and pure compound that does not interfere with the absorbance measurement of NADPH at 340nm, a full absorbance spectra of all the samples were measured (from 300nm-500nm). Seeing that enzyme activity in this assay was monitored by the decrease in absorbance at 340nm due to NADPH consumption as a

direct correlation of substrate turnover, the absorbance reading of the samples had to be kept below a maximum of at least 0.7 optical density (OD). Preferably the absorbance value of the samples must be below or equal to 0.5 OD where NADPH absorbs at the concentrations typically used in the disulfide reductase assays.

A co-solvent mixture of assay buffer (50mM HEPES [pH 7.6], 1 mM EDTA) and DMSO (no more than 4%) of the samples were prepared to determine the sample absorption values to be used in the enzyme assays (Table. 4.1.).

**Table. 4.1. Absorbance measurement of sample concentrations at 340nm**

| Sample | Concentration (µg/mL) | Absorbance (OD 340nm) |
|---|---|---|
| *E. natalensis* | 50.00 | 0.49 |
| *K. vesicatoria* | 125.0 | 0.39 |
| *P. sidoides* | 200.0 | 0.56 |
| Compound 1[a] | 200.0 | 0.51 |
| Compound 2A[b] | 300.0 | 0.49 |
| 7MJ | 100.0 | 0.40 |

[a] stigmasta-5,23-dien-3-ol

[b] *5-(hydroxymethyl)furan-2(5*H*)-one*

### *4.2.2. Enzyme activity*

Glutathione disulfide reductase (Gtr) from baker's yeast (G3664), oxidised glutathione disodium salt (GSSG; G4626) and NADPH in tetrazolium salt (N1630) were all purchased from Sigma. Screening of samples on Gtr activity was carried out as microtitre plate assays in 96-well plates on a PowerWave XS microplate reader from Bio-Tek®. The assay mixture had a total volume of 200 µL and contained Gtr at 2.0 µg prot/mL, GSSG at 200 µM, 50 mM HEPES (pH 7.6), 1 mM EDTA, 200 µM NADPH and sample to be tested. The positive control contained Gtr at 2.0 µg prot/mL, GSSG at 200 µM, 50 mM HEPES (pH 7.6), 1 mM EDTA, 200 µM NADPH while the negative control consisted of only Gtr at 2.0 µg prot/mL, 50 mM HEPES (pH 7.6) and 1 mM EDTA, 200 µM NADPH. Reagent concentrations of the Gtr enzyme screening assays is summarised in Table 4.2. The conversion of NADPH to NADP by Gtr was measured at 340nm at 30°C for an average of 20min.

Sample stock solutions were made up using co-solvent mixtures of assay buffer and DMSO such that final assay mixtures contained no more than 2% DMSO. To minimize experimental error, the same percentage of DMSO was maintained in all assays carried out for each control and sample being tested.

Assays were carried out in triplicate on two separate occasions. Kinetic data analyses were performed using KCjunior v1.41.3 (Novell Software Ltd). Enzyme activity is expressed as the rate at which NADPH (which absorbs at 340nm) is oxidised to NADP (which does not absorb at 340nm), which directly correlates to the amount of substrate (GSSG) being reduced to product (GSH). The rate is expressed as mOD/min. The effect of the tested samples on the Gtr enzyme is expressed as the percentage enzyme activity (the rate of NADPH consumption) compared to the positive control.

**Table. 4.2. Reagent concentrations of Gtr enzyme screening assays for extracts and pure compounds**

| **Reagents** | **Samples** | **Controls** | |
|---|---|---|---|
| | | **Positive** | **Negative** |
| NADPH | 200 µM | 200 µM | 200 µM |
| GSSG | 200 µM[a] | 200 µM | No substrate added[b] |
| Samples | Absorbance[c] | No extract added[b] | |
| Gtr | 2.00 µg/mL | 2.00 µg/mL | 2.00 µg/mL |

[a]GSSG Km determined for Gtr

[b]Additional assay buffer added for total volume of 200 µL

[c]Highest concentration obtained with $OD_{340} \approx 0.5$

## 4.3. Results and discussion

The effect of the selected samples on Glutathione enzyme activity is summarised in Figure 4.5. and Table 4.3. The investigation on the GSSG reductase activity of Gtr, found that the whole extracts of *K. vesicatoria* (EtOH) failed to exhibit any significant activity at a concentration of 125 µg/mL. Even though the activity of the enzyme was not reduced with *E. natalensis* ($CHCl_3$) at a concentration of 50.0 µg/mL, it should be noted that approximately 20% of the NADPH was not oxidised to NADP, as determined by the OD after 20 min of running the assay.

In the case of the *K. vesicatoria* extract, all of the NADPH was converted to NADP by the enzyme during this time. This situation reflects the true nature of the sample's effect on Gtr, since the amount of NADPH can be directly compared to the amount of substrate. In the case of *E. natalensis*, only 80% of the substrate was converted to product by the enzyme, this either indicates a slight inhibitory activity of the extract on the enzyme, or that the extract contains substances that serve as alternative substrate for the enzyme. The latter is the most likely scenario as *E. natalensis* contains naphthoquinones that are known to serve as subversive substrates for Gtr. The most noteworthy activity was seen for the extract of *P. sidoides* (EtOH), with the enzyme only exhibiting 2% of its optimal activity (when treated with 200 µg/mL of the extract) and approximately 90% of

the substrate was left unused. *Pelargonium* is known to contain a variety of polyphenolic compounds and the presence of coumarins and tannins can explain the significant inhibitory activity of the extract on Gtr.

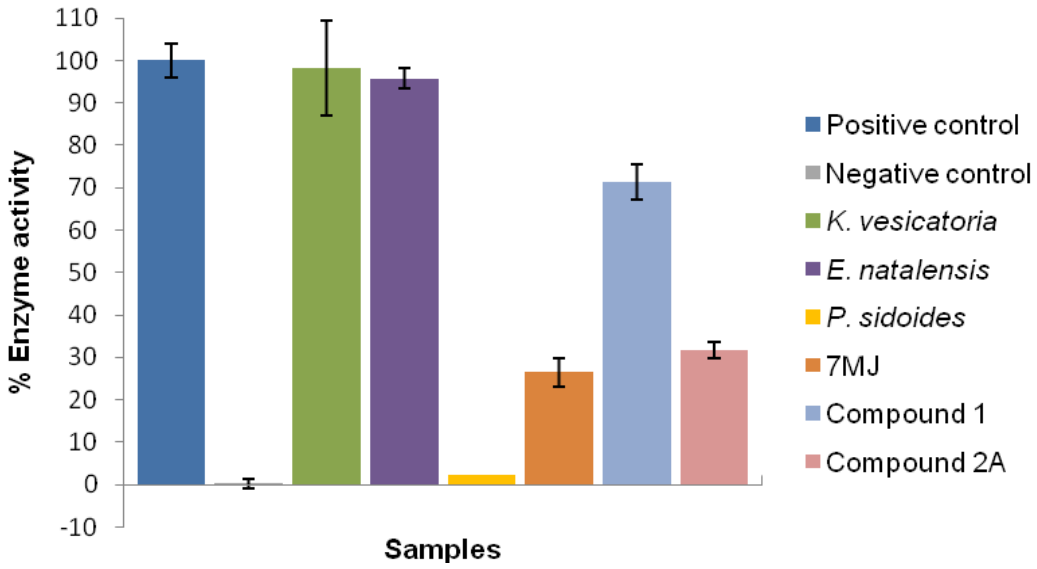


**Fig. 4.5. Enzyme activity of samples on Glutathione reductase**

**Table. 4.3. Inhibitory or subversive substrate activity of test samples on Gtr**

| Sample (µg/mL) | Gtr rate ± SD (%)[a] | NADPH (%)[b] | Activity |
|---|---|---|---|
| Positive control | 100.0 ± 3.900 | 100 | - |
| Negative control | 0.202 ± 0.025 | 0.00 | - |
| *E. natalensis* (50) | 95.78 ± 2.384 | 20.0 | Subversive |
| *K. vesicatoria* (125) | 98.28 ± 11.243 | 100 | No activity |
| *P. sidoides* (200) | 2.297 ± 0.005 | 0.00 | Inhibitory |
| Compound 1 (200) | 71.35 ± 4.056 | 100 | Inhibitory |
| Compound 2A (300) | 31.75 ± 1.857 | 0.00 | Inhibitory |
| 7MJ (100) | 26.49 ± 3.364 | 50.00 | Subversive |

[a]Enzyme rate (mean ± SD) expressed as percentage of the positive control 179.2 ± 7.15 mOD/min

[b]Approximate amount of NADPH converted to NADP

Misleading results are seen for the effect of 7-MJ on Gtr, as only 50% of the substrate was converted by the enzyme which again indicates that 7-MJ is either inhibiting the enzyme or serving as a subversive substrate. It is known that 7-MJ has futile substrate properties and as a naphthoquinone, it produces reactive oxygen species via redox cycling and the interaction with nucleophilic biomolecules, such as protein thiols or glutathione (Klaus *et al*., 2010). The subversive effect of 7-MJ can be clearly illustrated with a time-dependant assay where the reaction is

measured for at least 60min and not 15-20min as per usual (Labuschagné, 2008). The actual effect of the samples on Gtr activity is summarised in Table 4.3. Considering the activity of the isolated compounds from *K. vesicatoria*, Compound 1 (stigmasta-5,23-dien-3-ol) exhibited slight inhibitory activity on Gtr at a concentration of 200 µg/mL. The enzyme operated at 71% of its optimum rate, although 100% of the NADPH was depleted. This indicates that the enzyme is most likely inhibited by the sterol in a reversible manner. Gtr has a higher affinity for the substrate (GSSG) than for the sterol, slowing down the rate but not incapacitating the enzyme totally. Compound 2A (*5-(hydroxymethyl)furan-2(5*H*)-one*) had notably higher inhibitory activity on Gtr at a concentration of 300 µg/mL. The lactone inhibited the activity of the enzyme by approximately 70%, this percentage is an accurate estimation since none of the NADPH was oxidised, reflecting irreversible inhibition of the enzyme.

## 4.4. Conclusion

The investigation on the NADPH oxidase activity with Gtr, found that *K. vesicatoria* failed to exhibit any NADPH oxidase activity at 125 µg/mL concentration. Gtr is evidently not the target for the antitubercular activity of this extract. The isolated lactone, (*5-(hydroxymethyl)furan-2(5*H*)-one*) exhibited pronounced inhibition of Gtr. The absence of a correlation between the Gtr activity of this compound and the low antimycobacterial activity is probably due to the isolated condition of the enzyme assay and the high concentration of the compound (300 µg/mL). In addition to Mtr, mycobacteria have additional antioxidant defense enzyme systems such as thioredoxin, catalase/peroxidise and super oxide dismutase (SOD; Attarian *et al*., 2009) which in whole cell conditions could inactivate the inhibitory effect of the lactone. Indeed, the possibility of selective inhibition towards Gtr as compared to Mtr for the lactone is credible. The same reasoning can be applied to the inhibitory activity seen for the extract of *P. sidoides*, even though the extract contains polyphenols with noticeable inhibitory activity on Gtr, whole cell conditions in *M. tb* (and Mtr in itself) are markedly different.

The slight subversive substrate property of *E. natalensis* and its high antimycobacterial activity (with an MIC of 8.0 µg/mL) can be explained by the low amount of naphthoquinones in the crude extract and the ability of these compounds to accumulatively and non-specifically, react with multiple biological targets such as lipoamide dehydrogenase and thioredoxin reductase within *M. tb* bacilli. It has also been suggested that naphthoquinones could behave as non-functional ubiquinone and/or menaquinone surrogates, which may perturb electron transfer in respiratory chain processes (Patel and Blanchard, 2001). The apparent reversible inhibition of Gtr by Stigmasta-5,23-dien-3-ol could be more pronounced with Mtr and in part, explain for the antimycobacterial activity of this compound, although disruption of mycobacterial cell wall integrity is more likely to be the mechanism of action for this sterol.

Enzymes involved in the maintenance of mycothiol could make appealing drug targets and another possibility is to reduce the effect of endogenous mycothiol to inhibit enzymes involved in degrading the mycothiol-antibiotic complex. Since there is no exact mammalian equivalent to the mycothiol pathway it should be possible to achieve selective inhibition of the mycothiol biosynthesis. Previous results (Labuschagné, 2008) have already indicated the selective subversive substrate activity of 7-MJ and 8-chloro-5-hydroxy-7-methyl-1,4-naphthoquinone to Mtr. These two compounds exhibited Mtr activity of 700 and 500% compared to 40 and 50% for Gtr in time-dependant enzyme assays. Further investigations such as enzyme concentration- and time- dependant assays should be carried out for the crude extract of *E. natalensis* and both the isolated compounds (*5-(hydroxymethyl)furan-2(5*H*)-one*) and Stigmasta-5,23-dien-3-ol on Gtr and Mtr to establish selective subversive substrate and inhibitory properties.

By means of screening antimycobacterial samples for possible subversive substrates or inhibitory activity against Glutathione reductase, future tests against the mycobacterial enzyme (Mtr) can be planned and “fine tuned” and samples can be eliminated according to the Gtr screening results. Recombinant Mycothione reductase has to be purified from mycobacteria such as *M. smegmatis* transformants (Patel *et al*., 1999) and the substrate MSH is very hard to come by (Stewart *et al*., 2008).

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# CHAPTER 5
# CYTOTOXICITY, INTRACELLULAR ANTIMYCOBACTERIAL ACTIVITY and IMMUNOMODULATION of PLANT EXTRACTS, COMBINATIONS and COMPOUNDS

## 5.1. Introduction

### *5.1.1. Toxicity*

Toxicity screening in laboratory systems is a necessary aspect of the preliminary safety evaluation of plant-derived samples prior to further testing or compound isolation. This holds particularly true if further sample investigation involves test models such as cell-lines that are susceptible to specific thresholds of xenobiotics. As in this case, where intracellular inhibition of *M. tuberculosis* and cytokine levels of differentiated U937 (from monocytes to macrophages) cells are to be measured, it is necessary to establish the toxic threshold and to ensure that biological activity of the plant extract is not due to a general toxic effect. Employing a cell-line cytotoxicity assay, where viable macrophage cell growth after incubation with test compound is determined spectrophotometrically using a tetrazolium-based colorimetric assay (Mosman, 1983), establishes specific toxicity of the extracts on the differentiated monocytic U937 cell-line. The 50% inhibitory concentration ($IC_{50}$) values are calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

### *5.1.2. Immunomodulation*

Immunomodulation refers to the ability of many adjuvants to modify the cytokine network and, hence, the immune response. According to Schmutzler *et al.* (1989) the term “immunomodulation” includes at least three therapeutic goals- suppression, stimulation and restoration. For suppression clinically effective drugs like corticosteroids and cyclosporin are available. For stimulation, however, clinical success has been achieved only with macromolecules like BCG and MDP (muramyl dipeptide) which serve as adjuvants to enhance B-cell activity. Levamisole is a well known synthetic compound which was investigated as an immunostimulant on T-cell activity in controlled clinical trials (Renoux, 1980; Huskisson & Adams, 1980; Spearfico, 1980; Russell, 1980; Miller, 1980). Since its use was aimed at restoring a deficient immune system through non-antigen dependent stimulation, it can also be regarded as an immunorestorative agent.

In the absence of an acquired immune system such as that possessed by humans, plants instead rely on an enormous variety of small-molecule antimicrobials. Over 100,000 such compounds are synthesised by plants (Dixon, 2001). Wagner (1990) has reviewed plant derived natural products with immunostimulatory activity which includes alkaloids, quinones, terpenoids, phenolcarboxylic acids and high molecular mass compounds such as polysaccharides and glycoproteins. Of the latter, arabinogalactans from *Echinacea* species have significant *in vitro* and *in vivo* immunostimulant properties. Activity of these naturally derived immunostimulatory products is particularly evident against soil bacteria and the most common bacteria in soil come under the genera Bacillus, Clostridium, Corynebacterium, and Mycobacterium (Dixon, 2001).

Plant products have been shown to improve the immune response to TB both *in vitro* and in clinical trials. Picroliv, an iridoid glycoside fraction of *Picrorhiza kurroa*, selectively augmented human T cell responses to mycobacterial protein antigens (Sinha *et al*., 1998) and Zaitzeva *et al*. (2009) reported on the clinical use of Dzherelo (an over-the-counter phytoconcentrate of many plants) as an immunotherapeutic adjunct in the management of TB. Significant clinical and radiological improvements and clearance of *M. tb* took place in newly diagnosed TB patients at a higher rate when Dzherelo was used as an adjunct to standard, first-line anti-TB drugs than in patients on antituberculous treatment alone. The proportion of TB patients cured according to sputum culture and radiology was two- to four-fold higher among Dzherelo recipients. Furthermore, the combination therapy gave results after a much shorter period. A great number of plant-derived constituents enhance and/or activate host defensive immune responses, and these studies implicate macrophages as one of the primary cellular targets through which these substances induce immunomodulatory, antitumourigenic, wound-healing and other therapeutic effects (Schepetkin and Quinn, 2006). Adjuvants can target macrophages to allow antigens to associate directly with Class I MHC molecules on the cell surface to elicit Class I-restricted CD8+ cytotoxic T-lymphocyte responses. Particulate antigens tend to target macrophages, and macrophages can be recruited to the site of antigen deposition through the action of cytokines (Kit, 2008). Macrophage activation by plant constituents is thought to be mediated primarily through the recognition by specific macrophage receptors. Activation of these receptors leads to intracellular signalling cascades, resulting in transcriptional activation and production of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), Interleukin (IL)-1, IL-12, and interferon gamma (IFN-γ) (Schepetkin and Quinn, 2006).

#### 5.1.2.1. Th1 and Th2 immune responses

Despite the fact that tuberculosis is such a prevalent and widespread disease, the human immune response is usually very effective. Only 5% of persons infected by the tubercle bacillus develop clinically evident primary disease within 3 years of infection, and only a further 5% develop post-primary disease due to endogenous reactivation or exogenous re-infection years or decades later

(Grange, 1997). Thus, in the absence of an obvious immunosuppressive disorder such as HIV infection, the tubercle bacillus is non-pathogenic in 90% of those infected. The nature of the difference between this protected majority and the minority of persons who develop the disease mainly lies within the fact that the immune responses leading to protective immunity on the one hand and to tissue-destroying hypersensitivity and progression of disease on the other are qualitatively different. In effect, there are two types of immune responses against tuberculosis infection. The first response is associated with a Th2 cell profile which causes tissue damage and may, under some circumstances, have a minor protective role by isolating bacteria in necrotic foci. This effect is also known as the Koch phenomenon. Robert Koch (1891) demonstrated that the intradermal challenge of guinea pigs with whole *M. tuberculosis* organisms or culture filtrate, four to six weeks after the establishment of infection, resulted in necrosis at both the inoculation site and the original tuberculous lesion site. A similar phenomenon occurs in persons with active TB, in whom the purified protein derivative (PPD) test site may become necrotic. However, the immune response associated with the Koch phenomenon does not actively destroy tubercle bacilli and thus does not lead to sterilisation of the tissues. The second type of response is associated with Th1-type cells that induce a state of true immunity which may or may not be accompanied by nonnecrotising tuberculin positivity (the latter may merely indicate the extent of ongoing boosting by the relevant epitopes).

Knowledge of the immune cells and their various subsets has undergone an explosion in the past 10 years. The existence of two subpopulations of helper T cells (Th1 and Th2) was first described by Mosmann (1991). Understanding of how the immune response is controlled by delicate balances between these two distinct subsets of CD4+ lymphocytes, based on their cytokine profile, has increased dramatically in recent years (Bouic, 2002). Certain immune pathologies have now begun to be classified into categories depending on the phenotypic presence of these different cells. It has been known for many years that different microbes or antigens administered under different conditions elicit very different types of immune responses that are best able to eliminate these microbes or antigens. For instance intracellular bacteria, such as most mycobacteria, stimulate macrophages giving rise to delayed-type hypersensitivity (DTH) reactions, and include the production of opsonizing and complement fixing antibodies. In striking contrast, helminthic parasites induce eosinophilic inflammation and the production of IgE antibodies. These differences are likely to result from the production of functionally distinct cytokines produced by a number of immune and non-immune cells. T lymphocytes, especially CD4-positive cells (CD4+ T cells), play a central role in the immune response to protein antigens. These functions are mediated by the secretion of cytokines in a supposedly polarized fashion- classified as either a Th1- or Th2-type cytokines (Breytenbach *et al*., 2001). Th1 and Th2 CD4+ T cells are characterized exclusively by differences in cytokine expression: Th1 cells are associated with IFN-γ, IL-2 and IL-12 secretions aswell as T cells involved with strong DTH reactions (Kit, 2008 and Romagnani, 1994). Th1 cells

also secrete TNF, thereby enhancing B-cell responses that elicit IgG2a, the antibody subclass most efficient in binding the serum complement proteins that enhance antigen-antibody reactions (Kit, 2008). Th2 cells express IL-4, IL-5, IL-6, IL-10 and IL-13 (Romagnani, 1995 and Harris *et al*., 2007) that activate the production of high levels of IgG1, IgA, and IgE by B cells (Kit, 2008). Th1 is primarily concerned with cellular immunity and inflammation, whereas Th2 is primarily concerned with humoral immunity. The dichotomy between Th1 and Th2 has been identified in murine CD4+ T cells (Mosman and Coffman, 1989) and the analysis of T cell clones in humans has shown an analogous, although not identical, cytokine synthesis heterogeneity (Romagnani, 1994). This cytokine heterogeneity is not restricted to CD4+ T cells, as other cell types also contribute to the secretion of regulatory cytokines. Thus, the terms Th1-type and Th2-type cytokine or cells are used to characterize the cytokine profile of different cell types. Armed with this knowledge, specific subsets can now be targeted and hopefully restore the delicate balance desperately required to bring relief to chronic conditions such as TB.

The cytokines produced by one type inhibit the other maturation pathway and thus, each of these classes of T helper cells antagonises production of the other (Grange *et al*., 1994). As a result, an immune response tends to become ‘locked in’ to one or other pattern of response. In most cases, an infection elicits a protective response, but if for some reason the host is locked in to an inappropriate response, the response itself contributes to tissue damage and progression of disease. There is now ample evidence that a Th1 response is required for protection against tuberculosis, whereas a Th2 (or mixed Th1 and Th2) response leads to tissue damage resulting, for example, in extensive pulmonary cavitation, weight loss and wasting (Grange, 1997). During the Th1 response, TNF-α acts as macrophage-activating factor. If, however, there is a mixed Th1 and Th2 response, the inflamed tissue becomes extremely sensitive to TNF-α, and release of the cytokine causes necrosis, first involving the microvasculature and subsequently the whole tissue (Grange and Stanford, 1995).

Recent studies suggest that although *M. tb* induces Th1 IFN-γ cytokine secretion, active TB is characterized by relatively high levels of Th2 cytokines, particularly IL-4. The resulting low ratio of IFN-γ/IL-4 skews the immune system away from a protective Th1 response (Demissie *et al*., 2006 and Wassie *et al*., 2008). Once the disease process of TB is established, endocrine changes (due in part to the effect of cytokines on the adrenal gland) further enhance Th2 maturation and progression of the disease and the subversive effect of the Th2-like response ultimately impairs bactericidal function. Potent immunosuppressive Th2 cytokines such as IL-4 and IL-10 may be involved, since they inhibit macrophage activity by down-regulating the Toll-like receptor 2 (TLR) pathway and inducible nitric oxide (iNOS) synthesis (Rook *et al*., 2004; Roy *et al*., 2008; Voskuil *et al*., 2003). Toll-like receptors are related to the fruit fly protein *Toll*; they enable cells of the innate immune system to recognize danger, for example, TLR4 binds bacterial endotoxin such as

lipopolysaccharide (LPS; Clark, 2008). Live strains of *M. tb* and their products, like lipid components, also elicit production of the Th2 cytokines (Harris *et al*., 2007).

The importance of cell-mediated immunity in the protective response against *M. tuberculosis* is well established (Antas *et al*., 2006). The production of cytokines such as interleukin-12 (IL-12) interferon gamma, (IFN-γ) and TNF-α is essential for macrophage activation, control of mycobacterial replication, and granuloma formation and maintenance in both mice and human beings (Flynn *et al*., 1993 and 1995; Stenger and Modlin, 1999). The production of IFN-γ and TNF-α, as well as other proinflammatory (Th1 type) and anti-inflammatory (Th2 type) cytokines is important in the innate immune response (Tosi, 2005; Nance *et al*., 2005; Schaible and Kaufmann 2000; Byrne *et al*., 2004). *Mycobacterium tuberculosis* has developed several strategies for exploiting distinct intracellular niches that enable survival and replication as well as protection from antibacterial activities of the host (O'Brien *et al.,* 1996; Russell *et al.,* 1997; Sinai and Joiner, 1997). The robust hydrophobic cell wall promotes mycobacterial replication within phagosomes of resting macrophages. This is further supported by the capacity of *M*. *tb* to arrest phagosome maturation at an early stage. In this way, mycobacterial phagosomes are not acidified and prevented from fusing with lysosomes (Kaufmann and Hess, 2000). Their seclusion in an intracellular hiding place seems to be the main reason effective vaccines against these bacteria have not yet been developed. Classical vaccines depend on specific antibodies as the protective principle, but these are not critical for defense against these intracellular bacteria. Rather, effective vaccines and other therapies against these pathogens need to induce strong Th1 responses (Hess *et al*., 2000). According to Grange *et al*. (1994) a Th1 immune response is protective during infection and a Th2 response leads to pathogenesis. Accordingly, recent immunological findings indicate that a therapeutic measure able to switch from a Th2 to a Th1 response would be of great benefit in TB (Grange, 1997). In this context, plant compounds that can induce the Th1 response and subsequently suppress the Th2 response by increasing or decreasing the related cytokines, can be a valuable adjuvant to current anti-TB treatments. Selection of the appropriate immunoregulatory adjuvant not only leads to an enhanced immune response but also determines the isotype of IgG, which other immunoglobulins are made, and how much CD4+-directed cell-mediated immunity is generated (Kit, 2008).

#### 5.1.2.2. Cytokines

Originally the term lymphokines was used to identify a family of hormone-like protein substances secreted by antigen-stimulated T-lymphocytes. These substances are not anti-bodies but nevertheless play an important role in the immune response because they control chemotaxis of neutrophils, maturation of T-cells, antibody production by B-cells, etc. When it was discovered that many other cell types, including monocytes, neurons, neurologlial cells, endothelial cells etc, secrete similar substances, terms like monokines (produced by monocytes), cytokines, interleukins

and even function-related names (e.g. tumour necrosis factor) were used. This led to considerable confusion because different names were sometimes used for one and the same substance. To solve this problem, it was recommended (van Papendorp and Claassen, 2002) that only the term interleukin (IL) be used to designate these different substances and that numerals be used to identify different interleukins, namely interleukin 1, 2, 3 etc (IL-1, IL-2, IL-3, etc). Cytokine is a more appropriate name and describes any of a class of soluble proteins (molecular weight less than 100,000 daltons, or 100 kDa) that are released by a cell to send messages that are delivered to the same cell (autocrine), an adjacent cell (paracrine), or a distant cell (endocrine). The cytokine binds to a specific receptor and causes a change in function or in development (differentiation) of the target cell (Clark, 2008).

Cytokines are involved in reproduction, growth and development, normal homeostatic regulation, response to injury and repair, blood clotting, and host resistance (immunity and tolerance). Many different types of cells can produce the same cytokine, and a single cytokine may act on a wide variety of target cells. Further, several cytokines may produce the same effect on a target, so the loss of one type of cytokine may have few if any consequences for the organism. Finally, the response of a target cell may be altered by the context in which it receives a cytokine signal. The context includes other cytokines in the milieu and extracellular matrix. Thus the concept of considering cytokines as letters of the alphabet that combines to spell words which make up a molecular language, is an easier way of understanding the complex milieu and functionality of cytokines.

Simply put, the immune system has many different types of cells acting together to take care of unwanted infections and altered cells. Cytokines are the protein messenger molecules produced by these cells in order to communicate and orchestrate the attack. Cytokines also maintain the normal growth, migration and survival of immune cells in a physiologic state. Just as hormones in the endocrine system can produce an effect on other cells, so cytokines can act on other immune cells, especially cells that are close by. Cytokines also act on non-immune cells, such as the blood vessel endothelium.

Cytokines may be divided into six groups: interleukins (IL), colony-stimulating factors (CSF), interferons (IFN), tumour necrosis factor (TNF), growth factors and chemokines. Interleukins are proteins that are produced primarily by one type of lymphocyte (a type of white blood cell, or leukocyte) or macrophage (a large phagocytic cell of the immune system) and that act on other leukocytes. However, it is now known that production of interleukins is not confined to lymphocytes and macrophages. Interleukins may be grouped into families with similar structure and function. The plethora of interleukins suggests considerable redundancy, although it is suspected that each interleukin may have some special purpose. So far, more than thirty-four types have been

described (Clark, 2008). The cytokines investigated in this study, and their functions, are summarised in Table. 5.1.

**Table. 5.1. Type Th1 and Th2 cytokines, sources and primary functions**

| Cytokine | Th1/Th2 type | Cellular Source | Principal actions |
|---|---|---|---|
| Interleukin-2 (IL-2) | Th1 | T lymphocytes, B cell lymphocytes, natural killer cells, oligodendrocytes | Proliferation of cytotoxic (killer) T lymphocytes and activated B-lymphocytes |
| Interleukin-4 (IL-4 or B cell-differentiating factor) | Th2 | T lymphocytes, mast cells, bone marrow stromal cells, foetal trophoblasts | Differentiation of B-lymphocytes, basophils, inhibits cell-mediated immunity |
| Interleukin-5 (IL-5, T cell replacing factor, B cell growth factor II, eosinophil differentiation factor, IgA enhancing factor) | Th2 | T lymphocytes, B lymphocytes, mast cells, eosinophils | Activation, growth and differentiation of B cells, eosinophil growth and differentiation, eosinophil chemotaxis, basophil activation |
| Interleukin-10 (IL-10 or (CSIF) cytokine synthesis inhibitory factor) | Th2 | T cell lymphocytes, B cell lymphocytes, macrophages, skin keratinocytes | Inhibits IFN-γ secretion by T cell lymphocytes and mononuclear cell inflammation |
| Interleukin-12 (IL-12) | Th1 | Macrophages, dendritic cells, B lymphocytes, T lymphocytes | Fosters formation of Th1-cells, enhances cytotoxic activities of NK-cells and T-cells and production of IFN-γ (by inducing gene expression in T lymphocytes and NK cells) |
| Interferon gamma (IFN-γ, interferon-type II or immune interferon) | Th1 | T-lymphocytes, NK cells | Activates macrophages and NK cells, activates specific genes, antiviral, activates neutrophils and macrophages to produce superoxide and nitric oxide |
| Tumour necrosis factor alpha (TNF-α or cachectin) | Th1 and Th2 | Macrophages | Activates NK cell cytotoxicity, enhances cytotoxic T lymphocytes production, activates production of INF-γ Fever, lysis of bone, haemorrhagic necrosis in tumours |

*5.1.2.2.1. Interleukin-2*

Interleukin-2 is a glycoprotein produced by the Th1 subset of T lymphocytes/T-helper cells. IL-2 may also be produced by macrophages, B cells, natural killer cells, and oligodendrocytes (which myelinate nerve cells) in the brain. IL-2 stimulates the development of cytotoxic (killer) T lymphocytes and activated B lymphocytes (which develop into antibody-secreting cells). IL-2 also activates non-specific (unlike T and B cells) cytotoxicity in natural killer (NK) cells. Natural killer cells participate in the early defense response to infection and may reject certain types of cancers

early in their development. IL-2 may also be made by foetal trophoblast cells that lie at the feto-maternal interface in the placenta. Receptors for IL-2 are found on lymphoid cells and on astroglia of the central nervous system. Johnson *et al*. (1995) have administered subcutaneous IL-2, at 12.50 pg twice daily for 30 days to South African patients with longstanding, treatment-refractory MDR-TB. With this short period of IL-2 administration, they have shown augmentation of NK and T-cell number and activation, rapid sputum conversion and improvement in chest radiographs. The clinical improvements persisted at least 4 months after IL-2 administration and there were no adverse events at this dose of lL-2. Intermittent intravenous IL-2 has been used successfully to increase CD4+ T-cell numbers in AIDS patients (Kovacs *et al*., 1996), a group at risk for pulmonary tuberculosis. Further studies need to determine the optimal route, dose and duration of IL-2 therapy. However, the available data strongly indicate an important therapeutic role for IL-2 in the treatment of mycobacterial diseases.

#### *5.1.2.2.2. Interleukin-4*

Interleukin-4, also known as B cell-differentiating factor (van Papendorp and Claassen, 2002) is a product of Th2 T cells, but may be produced by non-lymphoid cells such as foetal trophoblast cells that form the placenta. IL-4 promotes antibody responses by B lymphocytes, differentiation of basophils, inhibits cell-mediated immunity and NK cell activation by IL-2, and contributes to granuloma formation (possibly by acting on macrophages and endothelial cells). Granulomas are largely composed of macrophages and represent a particular type of chronic inflammation that can lead to tissue scarring and, hence, disease (Clark, 2008).

#### *5.1.2.2.3. Interleukin-5*

Interleukin-5, also known as T cell replacing factor (TRF), B cell growth factor II (BCGF-II), eosinophil differentiation factor and IgA enhancing factor (Hamblin, 1993). Interleukin-5, another Th2-type cytokine, promotes the development of B cells which ultimately produce immunoglobulin E, a class of antibody involved in immune responses to certain parasites and in allergies such as asthma and hay fever. IL-5 may arise from eosinophils and basophils (a type of granulocyte) and also stimulates the bone marrow to produce eosinophils, which are involved in allergies; eosinophils contain a 12-kDa major basic protein which is highly toxic to certain parasites (Clark, 2008).

#### *5.1.2.2.4. Interleukin-10*

Interleukin-10, originally known as cytokine synthesis inhibitory factor (CSIF), is produced by the Th2 subset of T cells as well as by B lymphocytes, macrophages, skin keratinocytes, nonepithelial nonleukocytic cells (stroma) in the endometrium, and foetal trophoblasts (Clark, 2008). IL-10 inhibits secretion (and function) of proinflammatory macrophages and activation of vascular endothelium and polymorphonuclear leukocytes (eosinophils, basophils, and neutrophils). Hence, inflammation and blood clotting are suppressed. IL-10 protects against lethal shock triggered by

bacterial endotoxin in sepsis by inhibiting production of proinflammatory (Th1) cytokines and other factors that contribute to low blood pressure (Clark, 2008). Inhibition of cytokine synthesis, especially of IFN-γ, by IL-10 can be greater than 90% but complete inhibition is not obtained. This may be due to the kinetics of action as IL-10 shows little or no inhibition of cytokine production before about 8 hours, and then inhibits synthesis very effectively from this time onward. Cytokines, such as IFN-γ, that are synthesized over a prolonged period are thus inhibited to a greater extent than cytokines synthesized within the first 8 hours after stimulation. In contrast to its ability to inhibit cytokine synthesis, IL-10 does not affect the antigen-stimulated proliferation of Th1 clones, provided that exogenous IL-2 is supplied (Mosmann and Moore, 1991). The exact mechanisms behind mycobacterial persistent infection and reactivation (the switch from latent to active infection) in tuberculosis remain poorly understood, although there is a possible role for the immunosuppressive cytokine IL-10 in triggering reactivation of disease (Freeman and Holland, 2007).

*5.1.2.2.5. Interleukin-12*

Interleukin-12 is a heterodimeric Th1-type cytokine composed of two chromosomally distinct components, p35 and p40, which comprise the bioactive form, p70 (Holland, 1996). IL-12 is produced primarily by antigen presenting cells (macrophages and some dendritic cells). The p35 form of IL-12 stimulates Th1 lymphocyte development, activates NK cells, enhances their proliferation and cytolytic activity. IL-12 is the major driver of T- and NK-cell INF-γ production and its production is stimulated by mycobacteria and mycobacterial products, especially in the setting of phagocytosis (Holland, 1997). IL-12 also stimulates NK cells to produce granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α as well as INF-γ, both of which are active *in vitro* and *in vivo* in the inhibition and killing of mycobacteria (Holland, 2001). The p40 form of IL-12 acts on macrophages. IFN-γ ligand binds to its receptor on the macrophage cell surface and activates the macrophage resulting in enhanced TNF-α production, production of other cytokines and chemokines, upregulation of MHC class II expression, enhanced antigen processing, and production of reactive oxygen and (in mice) nitrogen intermediates (Dorman and Holland, 2000). IL-12 may substitute for IL-2 in Th1-type responses (Clark, 2008). IL-12 plays a key role in promoting Th1 responses and subsequent cell mediated immunity.

*5.1.2.2.6. Interferon-gamma*

Interferons are a class of small protein and glycoprotein cytokines (15-28 kDa) produced by T cells, fibroblasts, and other cells in response to viral infection and other biological and synthetic stimuli. Interferons bind to specific receptors on cell membranes, their effects include inducing enzymes, suppressing cell proliferation, inhibiting viral proliferation, enhancing the phagocytic activity of macrophages, and augmenting the cytotoxic activity of T lymphocytes (Clark, 2008). Interferons classically interfere with the virus-replication mechanism within cells. These cytokines are divided into five major classes (alpha -α, beta -β, gamma -γ, tau -τ, and omega -ω) and several subclasses

(indicated by Arabic numerals and letters) on the basis of physicochemical properties, cells of origin, mode of induction, and antibody reactions (Clark, 2008). Interferon-γ activates NK cells and is a potent activator of macrophages. IFN-γ activates transcription of a large number of genes that play roles in antiviral activity, apoptosis, antigen processing, MHC protein expression, and Th1 cell development. IFN-γ also activates macrophages to kill or restrict growth of microbial targets, this function appears to be important in host defense against mycobacteria (Dorman and Holland, 2000). IFN-γ-induced generation of reactive nitrogen intermediates is one mechanism of *M. tuberculosis* killing in mice, however, reactive nitrogen intermediates have not been shown conclusively to play a major role in mycobacterial killing by human cells (Dorman and Holland, 2000).

Despite understanding of IFN-γ signal transduction, the pathways by which this cytokine activates mycobactericidal macrophage activities in humans are poorly understood. Interferon-γ activates neutrophils and macrophages to produce superoxide, increase surface display of MHC antigens and Fc receptors, decrease lysosomal pH, produce nitric oxide (NO) and increase the intracellular concentration of certain antibiotics (Holland, 1997). Despite the clear effects that IFN-γ has on phagocytes, its role in the control of mycobacteria remains controversial. IFN-γ in TB infected mouse models, suggested that its main effect was to enhance the acidification of mycobacteria-containing vesicles (Holland, 1996). Human *in vitro* systems have been more difficult to define. Several *in vitro* studies have shown that IFN-γ has both mycobactericidal and mycobacteriostatic activity in human macrophages (Bermudez and Young, 1988; Denis, 1991; Bermudez, 1993). The *in vivo* data on the role of IFN-γ in control and elimination of mycobacteria are compelling. Mice rendered incapable of IFN-γ production by gene targeting develop widespread mycobacterial infection with very poor granulomatous response and succumb rapidly (Flynn *et al*., 1993; Cooper *et al*., 1993). Exogenously supplied IFN-γ has not been able to restore normal mycobacterial resistance in these mice, suggesting that interferon-y may play a critical developmental role as well. Similar results were seen in IFN-γ receptor-deficient mice, clearly defining a necessary role for IFN-γ in mycobacterial resistance and control (Kamijo *et al*., 1993). However, it is also possible that some of the effect of IFN-γ is due to effects other than direct augmentation of the inhibitory effect of the phagocyte. Interferon-gamma might also improve or augment antigen presentation, leading to recruitment of CD4+ T lymphocytes and/or cytotoxic T lymphocytes, which may participate in mycobacterial killing (Condos *et al*., 1998).

#### *5.1.2.2.7. Tumour necrosis factor alpha*

Tumour necrosis factor alpha (TNF-α) or cachectin (van Papendorp and Claassen, 2002) is produced by a variety of cell types, but activated macrophages represent the dominant source. TNF-α activates NK cell cytotoxicity, enhances generation of cytotoxic T lymphocytes and activates NK cells to produce INF-γ (Clark, 2008). TNF-α also acts on vascular endothelium to promote

inflammation and thrombosis (formation of a blood clot in a blood vessel), these effects may be enhanced by the co-presence of IL-1 and INF-γ. TNF-α may also induce apoptosis (programmed cell death), and this also may be enhanced by the co-presence of INF-γ (Clark, 2008).

Indeed, a synergistic effect of TNF-α and IFN-γ has been described in a variety of disorders and in some cases for the cytokines to act, a TLR signal is also required. At low levels, TNF-α may serve as a growth and differentiation signal to trophoblasts and other cell types. Soluble receptors to TNF-α may bind and neutralize biological function (Clark, 2008).

However, TNF-α does not play a strictly beneficial role in tuberculosis pathogenesis: increased plasma TNF-α levels have been associated with clinical deterioration early in the treatment of severe tuberculosis. TNF-α is the cytokine widely thought to be responsible for the effects of tuberculosis including fever, weight loss, tissue necrosis and the Koch phenomenon (Holland, 1996). Although it may have deleterious roles in the setting of overproduction, TNF-α has a critical role in the control of mycobacteria. TNF-α also concentrates antimycobacterial drugs within macrophages (Bermudez *et al*., 1991).

## 5.2. Materials and methods

### *5.2.1. Cytotoxicity assay*

#### 5.2.1.1. Differentiation of U937 cells to activated macrophages

The histiocytic lymphoma cell line U937 (ATCC available from Highveld Biological (Pty) (Ltd), (Sandringham, South Africa) was maintained in RPMI (developed at Roswell Park Memorial Institute) 1640 medium (pH 7.2), supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine and a 0.1% antimicrobial solution (pennicilin, streptomycin and an anti-fungal, fungizone). Reagents were procured from Highveld Biological (Pty) (Ltd) (Sandringham, South Africa). Subculture of the cells was done every 2-3 days after a confluent monolayer had formed. During subculture, cells that attached to the culture flask were trypsinized (0.25% trypsin containing 0.01% EDTA) for 10 min at 37$^{o}$C then stopped by the addition of complete medium. Cells were grown to a density of 5 x10$^{8}$ cells/mL, centrifuged and washed with phosphate buffered saline (PBS) solution. Three sets of cells (in triplicate) were either treated with medium only, 12-o-tetradecanoyl phorbol-13-acetate (TPA, 10.0 µg/mL) or phorbol 12-myristate 13-acetate (PMA, 0.10 µg/mL; Sigma) and left to attach to sterilised glass cover slips in 6-well culture plates for three days at 37$^{o}$C in a humidified atmosphere of 5% $CO_2$ to stimulate differentiation of the monocytes to mature macrophages (Passmore *et al*., 2001; Hosoya and Marunouchi, 1992). Differentiation was monitored for 3 days following the addition of the inducers and its degree was evaluated by morphological criteria. The treated cells were fixed in Bouin's fixative for 30 min after 24 and 72 hours of stimulation and subsequently stained via the haematoxylin and eosin cell staining

procedure (Lillie *et al*., 1977 and Ausubel *et al*., 1994). To determine the optimal inducer of differentiation, cells were viewed and images captured with a Nikon Stereo light microscope (Apo 1.4 lens).

#### 5.2.1.2. Macrophage cytotoxicity assay

U937 human monocyte cells were maintained as described in section 5.2.1.1. Cells were grown to a density of $5x10^8$ cells/mL, centrifuged and washed with PBS solution. The cells were then counted with a hematocytometer using a light microscope to establish the concentration of the cells and resuspended in the correct amount of supplemented (10% FCS, 2 mM L-glutamine, 0.1% antimicrobial solution) RPMI 1640 medium as calculated. The concentration of cells was thus adjusted to $10^5$ cells/mL in the complete medium containing a final concentration of 0.10 µg/mL PMA (5.2.1.1. and 5.3.1.). Two hundred microlitres of the cell suspension were seeded into the inner wells of a 96-well tissue culture plate, while the outer wells received 200.0 µl of incomplete medium. The cells were incubated for 24 hours at 37$^o$C in an atmosphere of 5% $CO_2$ to induce differentiation of monocytes to mature macrophages (Passmore *et al*., 2001; Hosoya and Marunouchi, 1992).

Cytotoxicity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide (XTT) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). Dilution series were made of the extracts of *E. natalensis*, *K. vesicatoria* (ethanol crude and tannin free) and *P. sidoides* at the various concentrations (400.0 to 3.125 µg/mL). Synergistic combinations were tested at MIC and sub MIC levels to maintain the correct ratios of each extract as tested against *M. tb*, accordingly each combination was tested at a different starting concentrations: Combination 1 was made up to a combined concentration of 5058.2 µg/mL in the first well with a final concentration of 39.52 µg/mL. Combination 2 was made up to a combined concentration of 5058.0 µg/mL in the first well with a final concentration of 39.52 µg/mL and Combination 3 with concentrations ranging from 50.20 to 0.392 µg/mL. The pure compounds (INH, stigmasta-5,23-dien-3-ol and 5-(hydroxymethyl)furan-2(5H)-one) were made up to a stock concentration of 20.00 mg/mL and serially diluted to start with a concentration of 200.0 to 1.562 µg/mL from the first wells to the last in the microtitre plates. The isomer mixture (Compound A and B) was treated in the same way. These dilutions were added to the inner wells of the microtiter plate and incubated for 72 hours. After 72 hours, 50.0 µL of XTT reagent (1.0 mg/mL XTT with 0.383 mg/mL PBS) was added to the wells and the plates were then incubated for 1-2 hours. The positive drug, (Actinomycin D; Sigma), at a final concentration range of $5.0 \times 10^{-2}$ to $3.9 \times 10^{-4}$ µg/mL, was included. After incubation, the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader (PowerWave XS, Bio-Tek), which measures the OD at 450 nm with a reference wavelength of 690 nm. DMSO (0.04 %) was added

to serve as the control for cell survival. GraphPad Prism 4.03 software was used to statistically analyse the 50% inhibitory concentration ($IC_{50}$) values.

### *5.2.2. Parallel intracellular MIC determination and cell supernatant harvesting for immune modulation*

#### 5.2.2.1. Determining the multiplicity of infection

The multiplicity of infection (MOI) is a measure of the ratio of infectious agents (*M. tb* bacilli) to the infection target (macrophage cells). The MOI is defined by the number of infectious particles divided by the number of target cells present (Condit, 2007). For evaluation of mycobacterial binding, Zeihl-Neelsen (ZN) staining and light microscopy was used. ZN staining was performed according to standard protocols (Bishop and Neumann, 1970). In brief, differentiated cells were infected with increasing amounts of *M. tb* (1, 3 and 5 bacilli or CFU's per cell) for 16 hours, heat fixed, then stained with carbol-fuchsin (Bacto TB Carbolfuchsin KF®, Becton-Dickinson) for 4 min, washed, and incubated with HCl until the stain was completely dissolved. Counterstaining was performed with brilliant green (Bacto TB Brilliant Green K®, Becton- Dickinson) for 20s. Plates were air dried and resin mounted after thorough washing. Mycobacterial binding was investigated via light microscopy with a Nikon Stereo light microscope (Apo 1.4 lens) to determine the optimal MOI.

#### 5.2.2.2. Preparation of cells

U937 cells were maintained as described previously (5.2.1.1.) and induced to differentiate by exposing the cells to a final concentration of 0.1 µg/ml of PMA (5.2.1.1. and 5.3.1.) for 24 hours. Hundred microlitres of mature macrophage cells (1 x $10^5$ cells/mL) were seeded into the inner wells of a 96 well microtiter plate, while in the outer wells 200.0 µl of incomplete medium was added.

#### 5.2.2.3. Preparation of mycobacteria

*Mycobacterium tuberculosis* H37Rv was maintained as previously described (section 2.4.4.2.). Three week-old colonies were scraped from LJ slants and a concentration of 3 x $10^5$ CFU/mL was prepared in 10% FCS RPMI medium to coat bacilli in serum-opsonins proposed by Passmore *et al*. (2001) to increase the ability of U937 cells to bind *M. tuberculosis*. In addition, a final concentration of 2.5% PANTA was present in the bacilli-medium mixture to prevent contamination by non-mycobacterial organisms.

#### 5.2.2.4. Preparation of sample solutions

Samples tested for their immunomodulatory activity included the extracts of *K. vesicatoria* (41.3, 50.0 and 25.0 µg/mL), *E. natalensis* (12.2, 8.00 and 4.00 µg/mL), synergistic Combination 3 (C3; 121, 6.28 and 3.14 µg/mL) and the pure compound 7-MJ (previously isolated from *E. natalensis*;

18.0, 0.5. and 0.35 µg/mL) at their $IC_{50}$, MIC and half MIC concentrations. The anti-tubercular drug INH (200, 0.20 and 0.10 µg/mL) was also included at these concentrations. Other samples included the extract of *P. sidoides* (43.0 µg/mL) and the isolated Compound 1 (stigmasta-5,23-dien-3-ol; 16.4 µg/mL) at their $IC_{50}$ concentrations as their MIC and half MIC concentrations would lead to loss of cell viability. The same set of samples with MIC and half MIC values less than their $IC_{50}$ value were included for determining the intracellular antimycobacterial activity, eliminating *P. sidoides* and Compound 1. Seeing that the $IC_{50}$ (41.3 µg/mL) and MIC (50.0 µg/mL) values for *K. vesicatoria* were relatively similar, it was decided to include the MIC of this sample. The highest concentration of DMSO present in the assay (0.25%) was included as a solvent control. To ensure for enough sample (two sets of samples for infected and uninfected macrophages each sample tested in triplicate and samples replaced 24 and 72 hours after the first sample addition) a volume of 4 mL for each sample concentration was prepared in RPMI medium containing 2.5% PANTA with a DMSO concentration of no higher than 0.25%. Sample solutions were prepared fresh on the first day of the experiment (designated as 0 hrs) and the remaining sample solutions were stored at 4°C until used again at 24 and 72hrs after initial sample addition. Seeing that the highest concentrations used never exceeded the $IC_{50}$ concentrations of the individual test samples, cell viability was not affected.

#### 5.2.2.5. *Mycobacterium tuberculosis* infection of macrophages and cell supernatant harvest

The plated differentiated cells were washed with PBS three times to remove the antibiotic containing RPMI medium. The cells were then infected with 200 µl of tubercle bacilli at a final concentration of $3 \times 10^5$ CFU/mL resulting in a ratio of three bacilli per cell as the determined MOI of 3 (5.2.2.1. and 5.3.3.). The macrophages were allowed to phagocytose the bacteria for 16 hours at 37°C in a humidified atmosphere of 5% $CO_2$. After the infection period the cells were washed with PBS an additional three times to remove any unphagocytosed bacilli. To serve as the growth control, designated wells containing untreated infected macrophages were then lysed with 0.25% (w/v) sodium dodecyl sulfate (SDS, Sigma), doing 10-fold serial dilutions ($10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$) and plating the lysate on 7H11 agar medium for viable count determinations of intracellular organisms. After the 16 hour phagocytosis, 200.0 µL of fresh medium containing the desired test samples was transferred to each macrophage containing well (replicates of three per treatment), the sample-medium mixtures were replaced at 24 and 72hrs and the culture was enumerated after lysing the macrophages with 0.25% SDS on day 5 (120 hrs). The lysed contents of each well were resuspended with a needle and syringe, and 100.0 µL was transfered to 7H11 agar medium for viable count determinations, serial 10-fold dilutions of the lysate ($10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$) was included. In addition, on days one (24hrs), three (72hrs) and five (120hrs), 200.0 µl supernatant of each treatment was transferred to sterile Eppendorf tubes and frozen at -70°C for cytokine analysis. An additional set of uninfected macrophages were handled and treated in identical

conditions to the infected set of macrophages, to investigate the immunomodulatory effects of the samples on the macrophages alone. Methods used for intracellular infection were adapted from Rastogi (1996), Lall *et al.* (2005) and Passmore *et al*. (2001).

#### 5.2.2.6. CFU enumeration

The conventional method involved plating of 10-fold serial dilutions ($10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$) of sample lysate directly on 7H11 agar plates in triplicate for each concentration of samples and controls. Only the MIC/FIC and half MIC/FIC concentrations of the tested samples were plated out. Samples with MIC values above their $IC_{50}$ value were not included. These plates were incubated at 37°C in 5% $CO_2$ atmosphere for 3-4 weeks followed by CFU enumeration via the colony counting system ColorQCount® (Spiral Biotech, Model 530). Appropriate positive and negative controls were also included.

### *5.2.3. Immunomodulation*

#### 5.2.3.1. Cytometric bead array analysis

It has been reported that *M*. *tuberculosis* can survive in macrophages by various mechanisms and that anti-TB drugs effective on macrophages are needed for the treatment of TB. It was therefore decided to investigate the cytotoxicity of the plant extracts on a U937 cell line and to test the intracellular activity of these extracts against U937 cells infected with *M*. *tuberculosis*. To establish the immune modulation of the plant extracts on these infected cells, the expressed cytokine profiles of the infected cells can be determined by flow cytometry. The cells were infected with *M. tuberculosis* by diluting a concentration of actively growing bacilli into the cell culture medium and incubating with the cells over sixteen hours. The extract samples were then added to verify antituberculous activity intracellularly and the resultant cell supernatants were analyzed to determine possible immune modulation by the extracts. Methods used for intracellular infection were adapted from Rastogi (1996), Lall *et al*. (2005) and Passmore *et al*. (2001).

Many cytokines are pleiotropic, possess overlapping functions and regulate the production of other cytokines. Consequently, the make-up of the cytokine milieu is often of greater importance than the actions of a single cytokine. The analysis and quantitation of cytokines in biological fluids and tissue culture supernatant has become a widely-used procedure in research and clinical laboratories, and is clearly important in furthering our understanding of many immunological functions (Carson and Vignali, 1999). In support of these needs, there has been a surge of methodologies that have been developed that include bioassays, enzyme- linked immunosorbent assay (ELISA), intracellular staining, ribonuclease protection assay (RPA), polymerase chain reaction (PCR), high throughput quantitative analysis using Liquid Chromatography-Mass Spectrometry (LC-MS), and serum protein fingerprinting for quantifying cytokines (Prabhakar *et al*., 2004). In recent years, several commercial vendors and independent laboratories have developed

multiplexed/bead-based arrays for measuring cytokines. The basic principle behind this technology involves beads being labelled with a distinguishable fluorophore that allows it to be assigned or gated to a particular region by the scanner. Antibodies, specific for the cytokine of interest, are covalently linked to beads of a unique fluorescent region. The combination of different beads allows the user to simultaneously measure various cytokines or proteins of interest. The major advantages in using the multiplexed approach are the efficient use of samples because of low sample volumes needed for analysis and concomitant measurement of multiple cytokines, reduced time and cost of analysis, increased sensitivity, and greater dynamic range (Prabhakar *et al*., 2004).

#### 5.2.3.2. Cytokine detection

Cell culture supernatants were thawed once and examined for IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α concentrations by multiplex cytokine array analysis performed by using the Cytometric Bead Array (CBA) method using the Human Th1/Th2 Kit II (BD-Biosciences) as specified by the manufacturer. IL-12 levels attempted with a commercial Flex-set (BD-Biosciences) assay run in parallel. All cytokine determinations were performed with the same lots of samples. The method used was based on procedures used by Antas *et al*. (2006), Cook *et al*. (2001), and Jimenez *et al*. (2005).

The cytometric bead array (CBA) technique is based on microparticles matched with antibodies, in which the particles are dyed to six different fluorescence intensities. The dye has a maximal emission wavelength of approximately 650 nm (FL-3). The particles were matched by a covalent linkage with an antibody against one of the six cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5 or IL-10). The Antibody (Ab) particles serve as a capture for each cytokine. These Ab-particles were used as a mixture and six separate cytokines were simultaneously detected. The cytokines were directly detected in the immunoassay using six different antibodies matched with a phycoerythrin (PE), which emits at 585 nm (FL-2). The PE-conjugated detector antibody is used to complete the sandwich and the medium intensity in FL-2 is proportional to the concentration of the cytokine in the sample, which is quantified from a calibration curve. An important characteristic of the assay system is that the calibrators, the Ab-bead reagent and the second Ab-PE antibody reagent are each made of the mixture of all six cytokines. Six standard curves (standard ranging from 0 to 5000 pg/ml) were obtained from one set of calibrators and six results were obtained on one test sample.

Mixed Capture Beads (prepared using the procedure described by the vendor) were mixed on a Vortex mixer before adding 50.0 µL to the appropriate assay tubes. Fifty microlitres of the Human Th1/Th2 PE Detection Reagent was then added to the assay tubes and 50.0 µL of the Human Th1/Th2 Cytokine Standard dilutions were added to the control assay tubes. Of each of the test sample 50.0 µL was added to the test assay tubes. The assay tubes were then incubated for 3

hours at room temperature away from direct exposure to light. During the incubation, the Cytometer Setup procedure was performed. After the incubation time, 1.00 mL of Wash Buffer was added to each assay tube and centrifuged at 200 × g for 5 minutes. The supernatant from each assay tube was carefully aspirated (not to disturb the bead pellet) and discarded. To resuspend the bead pellet, 300 µL of Wash Buffer was added to each assay tube and mixed on a Vortex for 3-5 seconds before plating the samples out in 96-well microtiter plates. Acquisition was performed with flow cytometry utilising the BD FACSArray bioanalyzer (Fig. 5.1.). Cytokines are involved in reproduction, growth and development, normal homeostatic regulation, response to injury and repair, blood clotting, and host resistance (immunity and tolerance). Many different types of cells can produce the same cytokine, and a single cytokine may act on a wide variety of target cells. Further, several cytokines may produce the same effect on a target, so the loss of one type of cytokine may have few if any consequences for the organism. Finally, the response of a target cell may be altered by the context in which it receives a cytokine signal. The context includes other cytokines in the milieu and extracellular matrix. Thus the concept of considering cytokines as letters of the alphabet that combines to spell words which make up a molecular language, is an easier way of understanding the complex milieu and functionality of cytokines.

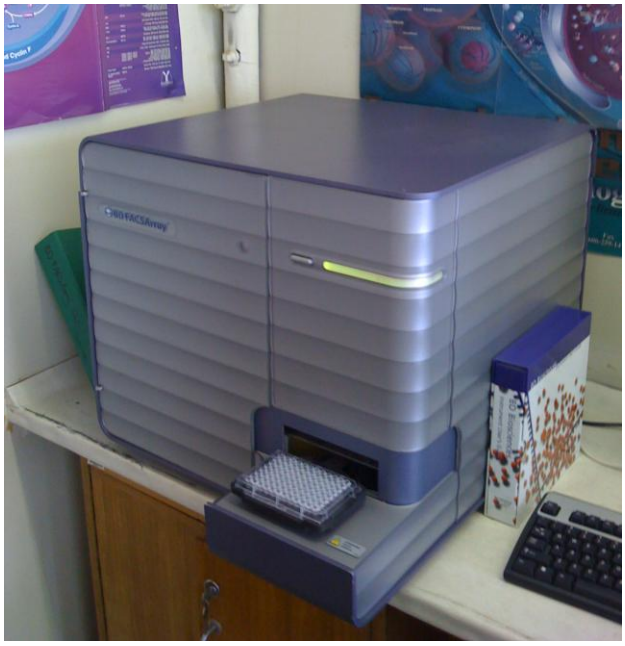


**Fig. 5.1. The BD FACSArray bioanalyzer.**

## 5.3. Results and discussion

### *5.3.1. Differentiation of U937 monocytes to mature macrophages*

U937 cells, a histiocytic lymphoma cell line, preserve all the monoblastic characteristics of *in vivo* monocytes, including the ability to differentiate into mature macrophages under the effects of different inducers (Hosoya and Marunouchi, 1992). U937 cells can be induced to differentiate

towards morphologically mature macrophage-like cells after treatment with phorbol esters (Lubert *et al*., 1991; Larsson *et al*., 1988). Phorbol esters such as PMA and TPA have pleiotropic effects on cells (Hass *et al*., 1989). Phorbol exerts its biologic effects by altering gene expression through the activation of Protein Kinase C (PKC; Pedrinaci *et al*., 1990) and modulating the activity of transcriptional factors such as Nuclear Factor kappa B (NFκB) that changes the expression of adhesion receptors (García *et al*., 1999).

Macrophages can be microscopically distinguished from monocytes by the presence of morphological changes. Undifferentiated cells grow in suspension (free-floating) and have a round shape, short microvilli, scarce cytoplasm and large bean-shaped nuclei. The cytoskeleton of differentiated U937 cells (macrophages) adapts accordingly to adhere to the growth surface (attached growth), assume an irregular flat shape with long pseudopodia and cytoplasmic projections and acquire phagocytotic capability (Pagliara *et al*., 2005).

After studying micrographs (Fig. 5.2.) of cells treated with PMA, TPA and medium only during a 24 and 72 hour period, macrophages were distinguished from monocytes by the presence of long psuedopods and cytoplasmic projections, lack of a smooth round shape and the lack of bean-shaped nuclei. It was evident that cells treated with medium only (Fig. 5.2.a.) were undifferentiated, as seen with the overall smooth round shape of the cells, lack of pseudopods, little cytoplasm and the presence of large bean-shaped nuclei. All these characteristics were present throughout the three day time period. Although treatment with TPA (Fig. 5.2.b.) resulted in the presence of cytoplasmic projections and long pseudopods from irregular shaped cells after the first day of incubation, the nuclei were still present as discernible bean-like shapes even after three days of treatment. These cells were notably smaller than medium and PMA treated cells. After only one day of incubation PMA treated cells (Fig. 5.2.c.) met all the criteria for macrophage morphology. Long psuedopods and cytoplasmic projections were present, the lack of a smooth round cell shape as well as the absence of bean-shaped nuclei. The size of these cells was also comparable to the healthy undifferentiated medium treated cells.

According to Pagliara *et al*. (2005) the most efficient molecule able to induce macrophage differentiation of U937 cells (as revealed by the large number of adhering cells, positive Nitro blue tretrazolyum (NBT) testing, the typical morphology of macrophages and latex particle phagocytotic capability) was TPA (50 ng/mL), followed in decreasing order by low glutamine concentration (0,05 mM/L), $Zn^{2+}$ (100mM/L) and DMSO (10%). In their study, differentiation of U937 cells induced by TPA started soon after the first day of incubation and increased progressively up to the third day.

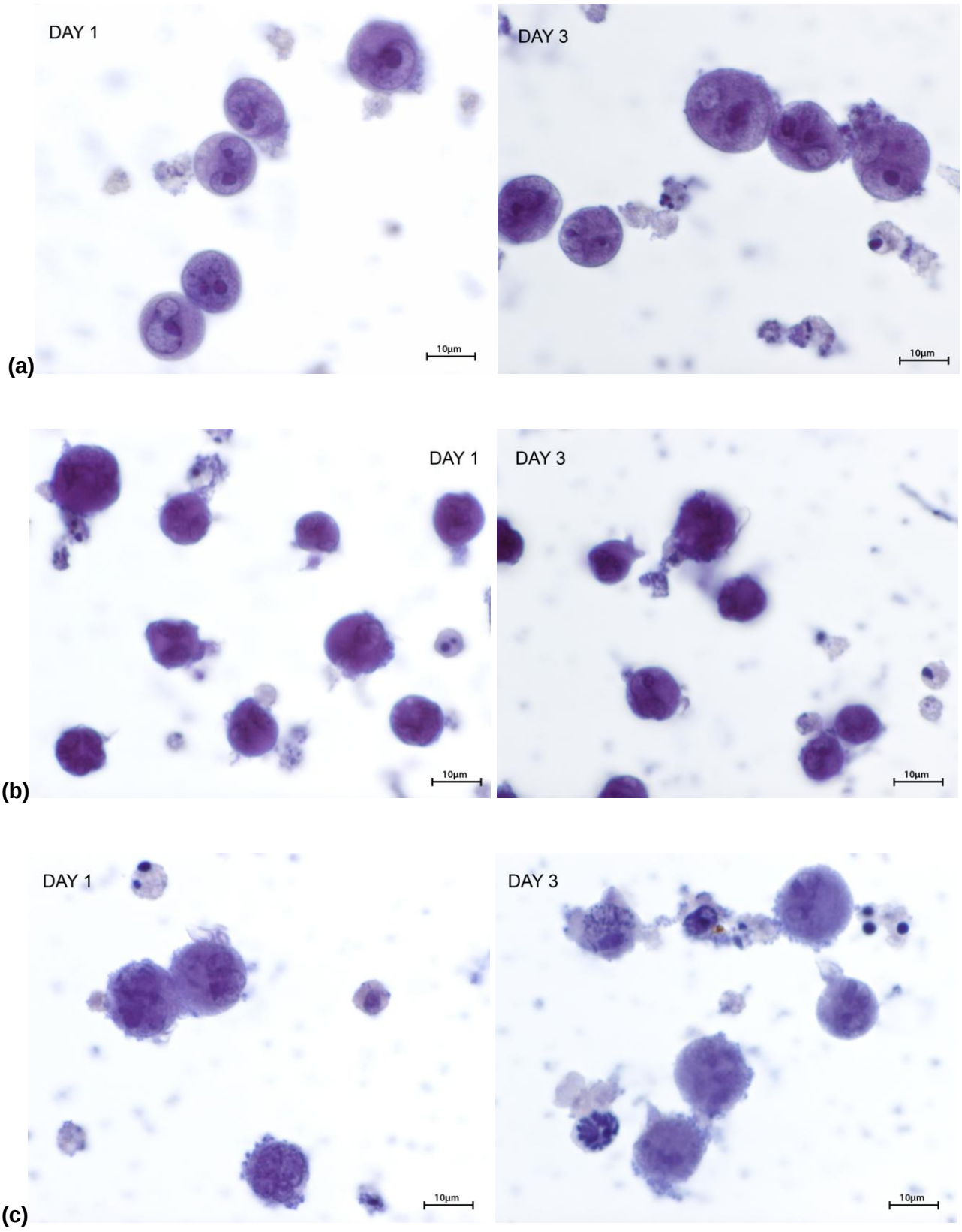



**Fig. 5.2. Light micrographs of hematoxylin/eosin stained U937 cells (100X magnification)**

**(a) Undifferentiated (medium only)**

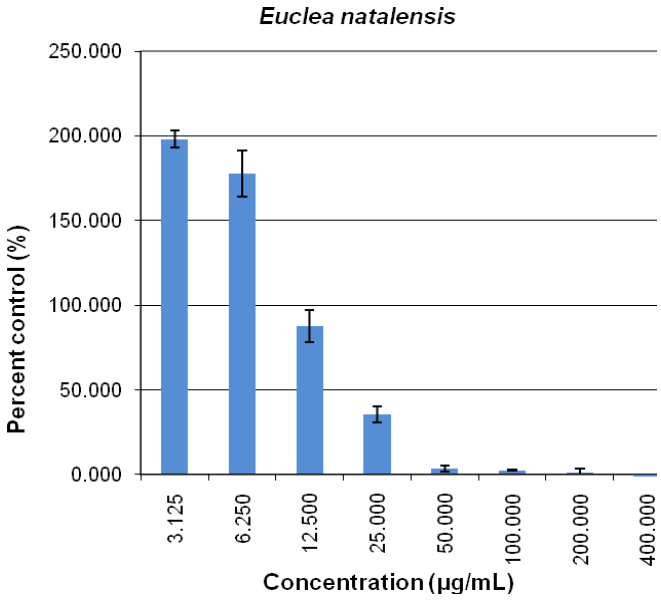
**(b)TPA differentiated and**

**(c) PMA differentiated**

According to a study done by Passmore *et al*. (2001) PMA-induced U937 cells showed the greatest ability to bind *M. tuberculosis* compared to Vit $D_3$ and IFN-γ stimulation and that Transmission Electron Microscopy (TEM) of PMA-treated U937 cells infected with *M. tuberculosis* revealed that the bacilli were intracellular and contained within vacuoles. It was decided to use a concentration of 0.10 µg/mL PMA as the differentiation inducer for further experiments due the presence of obvious macrophage morphology after only 24hrs and increased *M. tb* phagocytic activity as proposed by Passmore *et al*. (2001).

### *5.3.2. Differentiated U937 cytotoxicity assay*

The XTT cytotoxicity assay is a rapid and cost-effective tool to help choose optimal candidates, those samples with low cytotoxicity and high antimycobacterial activity, similar to a therapeutic dose, and to exclude any samples too toxic to test at their antimycobacterial concentration for intracellular assays.




**(a)**

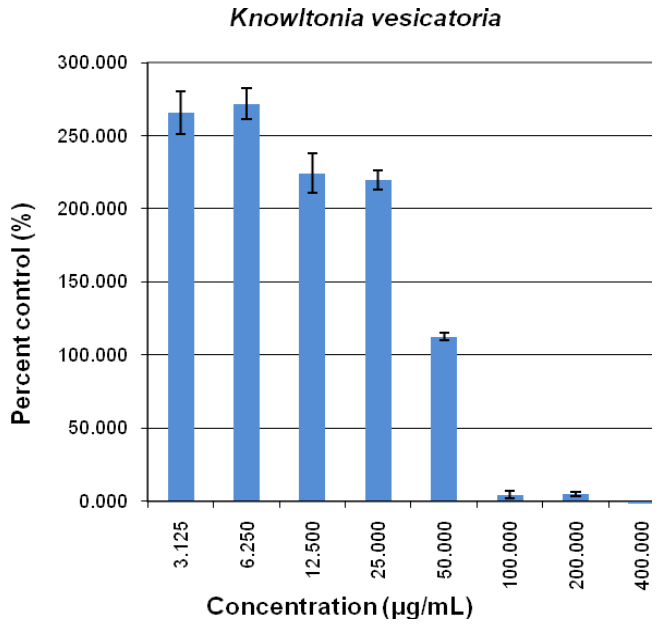
**Fig. 5.3. Cytotoxicity of extracts on U937 macrophages**

**(a) *E. natalensis* ($CHCl_3$)**

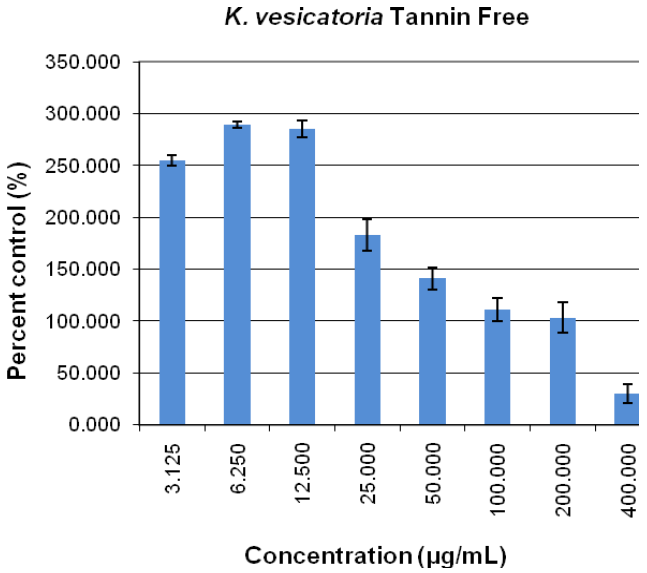
**(b) *K. vesicatoria* (EtOH)**

**(c) *K. vesicatoria*; tannin-free extract**

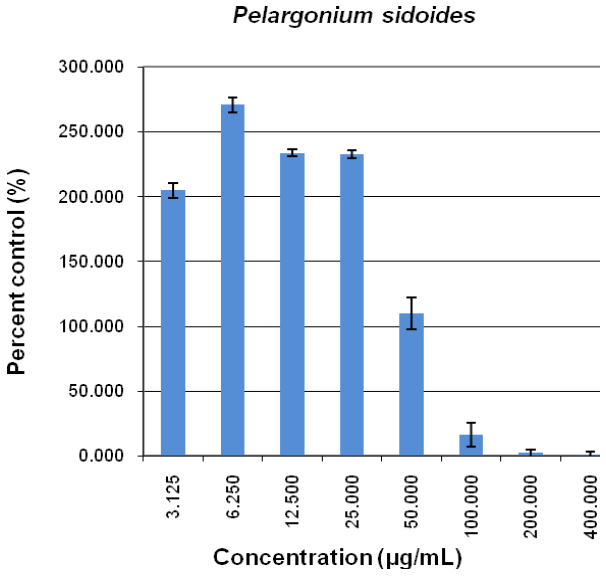
**(d) *P. sidoides* (EtOH)**




**(b)**



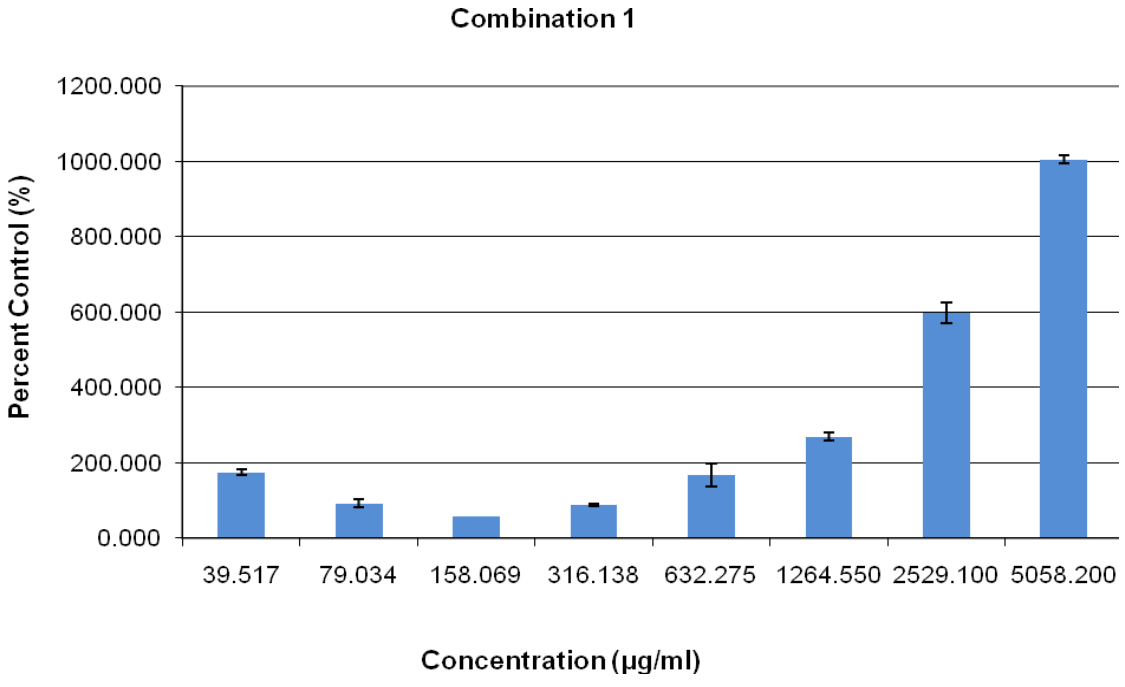

**(c)**



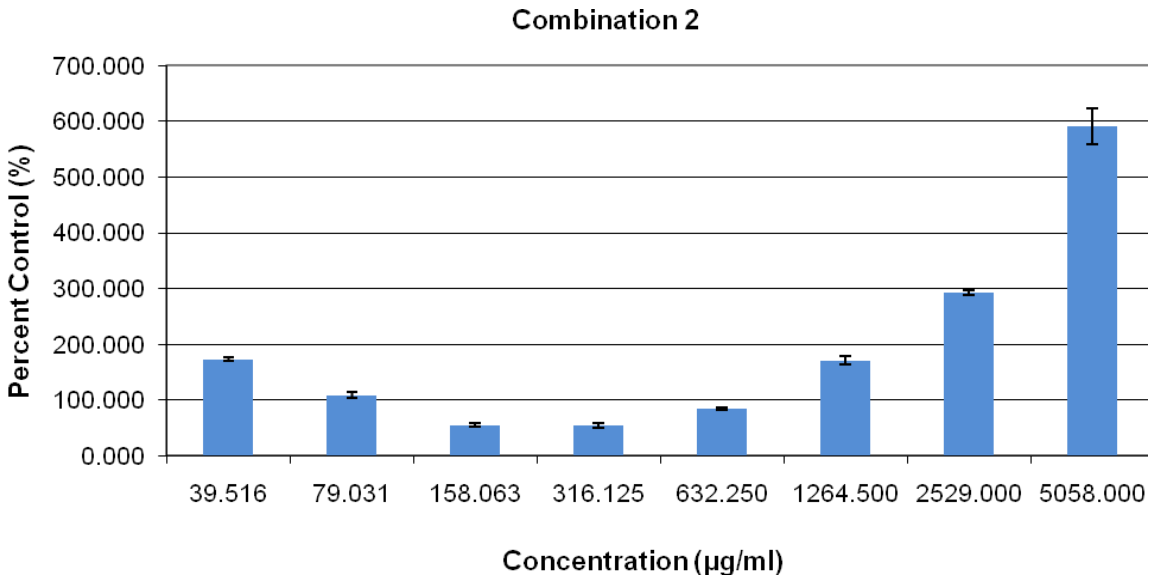

**(d)**

The results obtained (Fig. 5.3 and Table 5.2.) indicated that the cytotoxicity effects of the four plant extracts on U937 cells demonstrated marginal toxicity except for *E. natalensis*, which showed fifty percent inhibitory concentration ($IC_{50}$) at 12.22 µg/mL against the macrophages. The tannin-free extract of *K. vesicatoria* had the highest $IC_{50}$ value (64.77 µg/mL) compared to the other plant extracts, the lowered toxicity is most likely due to the lack of the protein binding polyphenols. *Knowltonia vesicatoria* and *P. sidoides* showed similar toxicity exhibiting $IC_{50}$ values at 41.25 and 43.54 µg/mL, respectively (Table. 5.2.).

With the synergistic combinations (Fig. 5.4. and Table 5.2.), especially Combination 1 and 2 which contained high concentrations of solutes and the *P. sidoides* extract, the calculated $IC_{50}$ values could not be correlated accurately with the graphs (Fig. 5.4.a. and b.) as the red extract of *Pelargonium* absorbed at the same wavelength as the XTT. Due to this unforeseen complication, the $IC_{50}$ could only be determined via the GraphPad Prism statistical software by excluding the higher concentration values. The approximate $IC_{50}$ values for Combination 1 and 2 were then calculated as 100.3 and 108.6 µg/mL. Synergistic combination C3 had a resultant $IC_{50}$ of 121.70 µg/mL. The toxicity profiles of the combinations were much better than the individual extracts indicating a synergistic action on lowering cytotoxicity. Although, as seen with the synergistic antimycobacterial activity of Combination 3, the synergism had a positive effect on the *K. vesicatoria* and a negative effect on the INH components of the combination. The $IC_{50}$ of *K. vesicatoria* was increased to 121.70 from 41.25 µg/mL and the cytotoxicity of INH decreased from above 200.0 µg/mL to 121.70 µg/mL.




**(a)**

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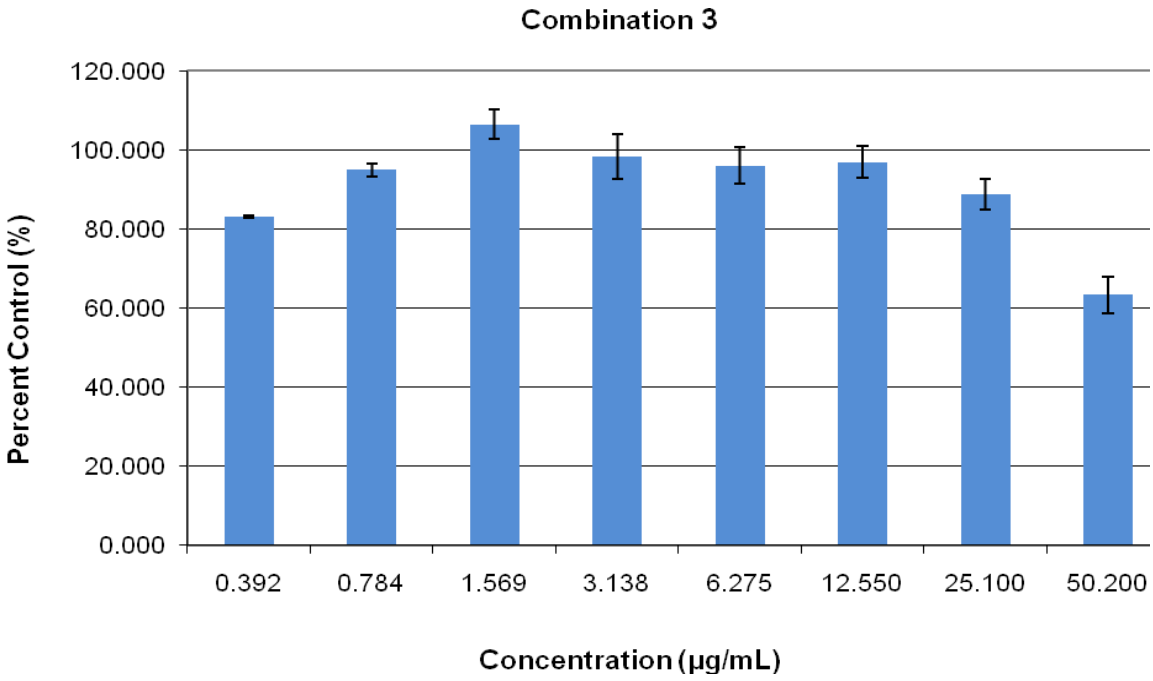



**(c)**

**Fig. 5.4. Cytotoxicity of the different synergistic combinations on differentiated U937 macrophages**

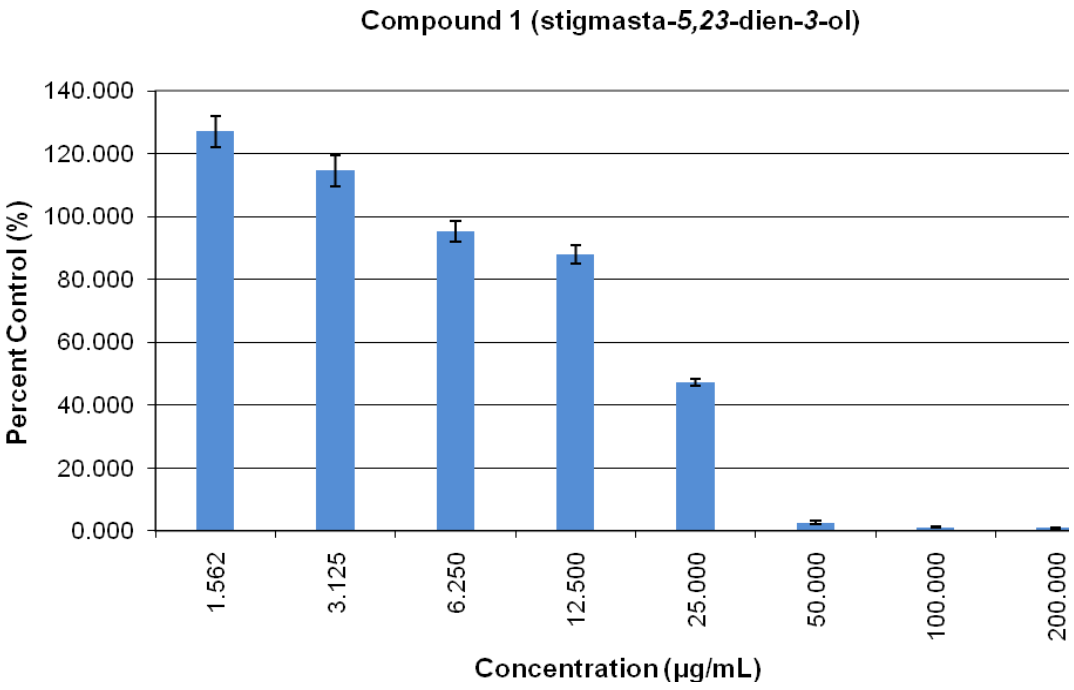
**(a) Combination 1: *K. vesicatoria*, *P. sidoides*, *E. natalensis* and INH**

**(b) Combination 2: *K. vesicatoria*, *P. sidoides* and *E. natalensis***

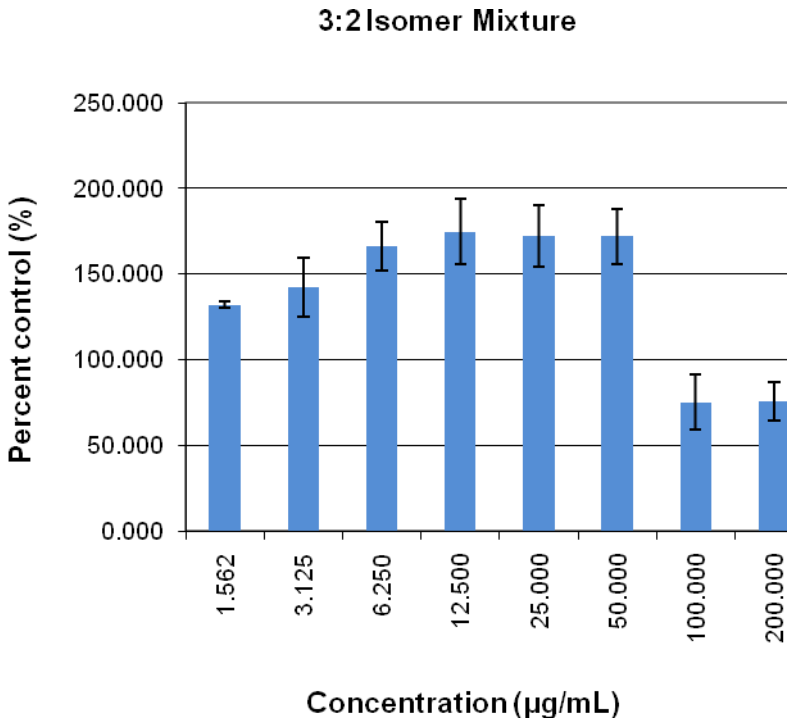
**(c) Combination 3: *K. vesicatoria* and INH**

The pure compounds (Fig. 5.5. and Table 5.2.), which included the anti-tubercular drug INH and the cytotoxic drug Actinomycin D, showed very different cytotoxic profiles compared to the extracts and combinations. With the compounds isolated from *K. vesicatoria*, Compound 1 (stigmasta-5,23-dien-3-ol) was more than two-fold as toxic to the cells when compared to Compound 2A (*5-(hydroxymethyl)furan-2(5*H*)-one*), with an $IC_{50}$ of 16.41 µg/mL and 44.70 µg/mL, respectively. The

3:2 isomer mixture of *5-(hydroxymethyl)furan-2(5H)-one* and *5-(hydroxymethyl)dihydrofuran-2(3H)-one* was not toxic to the macrophages even at the highest concentration tested (200.0 µg/mL) with more than 50 percent of the cells still viable (Fig. 5.5.c). Isoniazid had a similar effect on the cells (Fig. 5.5.d), with 80 percent of the cells still viable at 200.0 µg/mL, indicating an $IC_{50}$ above this value. Actinomycin D, which served as the positive control in the cytotoxicity assay, exhibited an $IC_{50}$ of 3.80 x $10^{-3}$ µg/mL.




**(a)**

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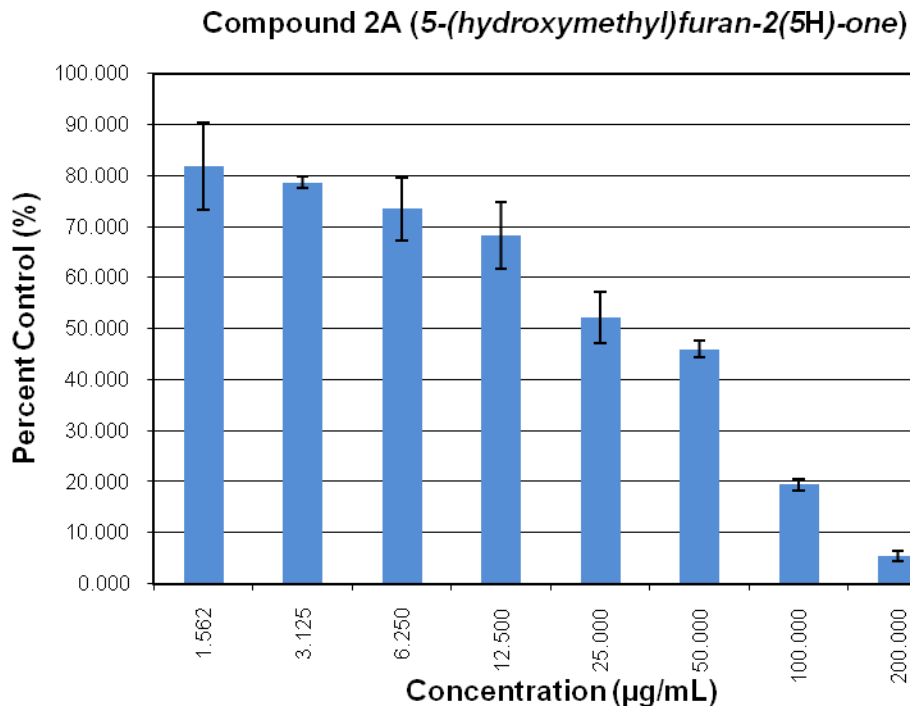


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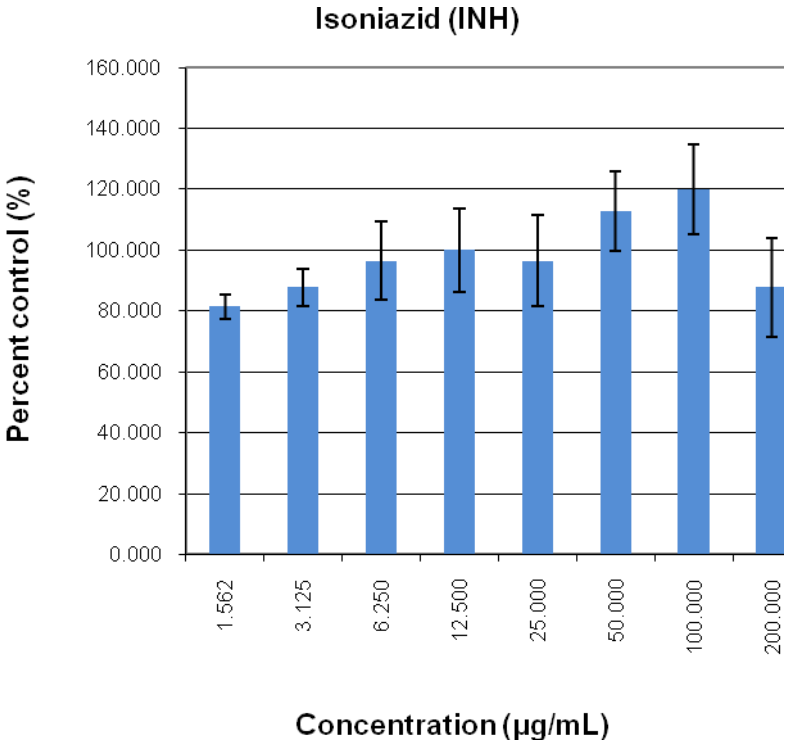
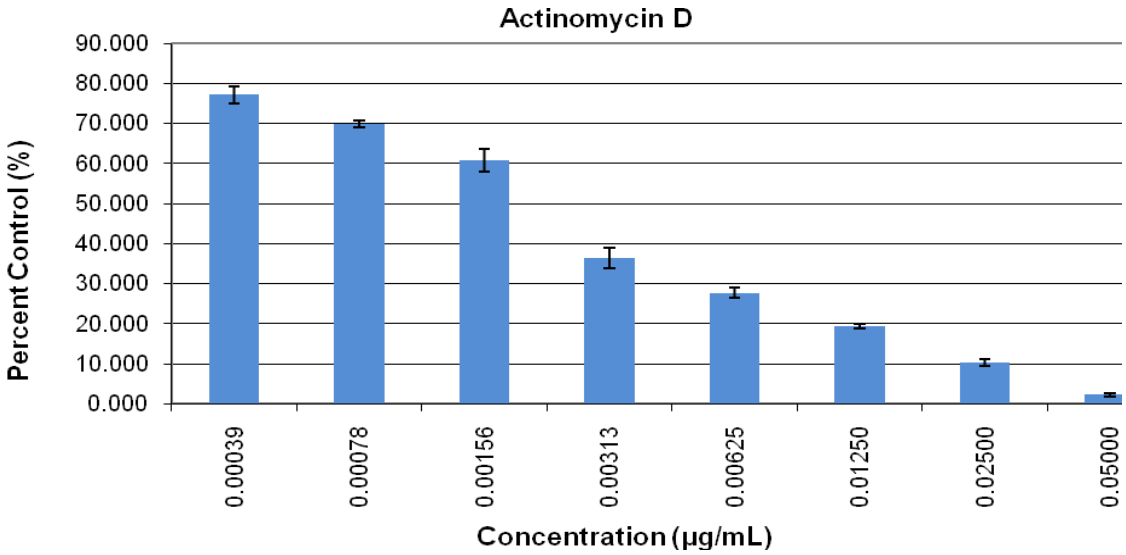



**(d)**




**(e)**

**Fig. 5.5. Cytotoxicity of compounds and positive controls on differentiated U937 macrophages**

**(a) Compound 1 (stigmasta-5,23-dien-3-ol)**

**(b) 3:2 Isomer Mixture (5-(hydroxymethyl)furan-2(5H)-one:5-(hydroxymethyl)dihydrofuran-2(3H)-one)**

**(c) Compound 2A (5-(hydroxymethyl)furan-2(5H)-one)**

**(d) Isoniazid (INH)**

**(e) Actinomycin D**

**Table. 5.2. Test sample cytotoxicity on U937 cells in comparison to antimycobacterial concentrations**

| Sample | $IC_{50}$ (µg/mL ± SD)[a] | MIC (µg/mL)[b] |
|---|---|---|
| *E. natalensis* | 12.22 ± 0.025 | 8.000 |
| *K. vesicatoria* | 41.25 ± 0.205 | 50.00 |
| *K. vesicatoria* (tannin-free) | 64.77 ± 1.812 | >2500 |
| *P. sidoides* | 43.54 ± 0.465 | 5000 |
| C1 | 100.3 ± 2.450 | 632.3 |
| C2 | 108.6 ± 0.89 | 2529 |
| C3 | 121.7 ± 2.079 | 6.275 |
| Isomer mixture | >200.0 | >200.0 |
| Compound 1 | 16.41 ± 0.135 | 50.00 |
| Compound 2A | 44.70 ± 0.50 | 200.0 |
| INH | >200.0 | 0.200 |
| Actinomycin D (positive control) | 3.820 ± 0.258 $x10^{-3}$ | - |

[a]Fifty percent inhibitory concentration

[b]Minimum inhibitory concentration

### *5.3.3. Determining the multiplicity of infection*

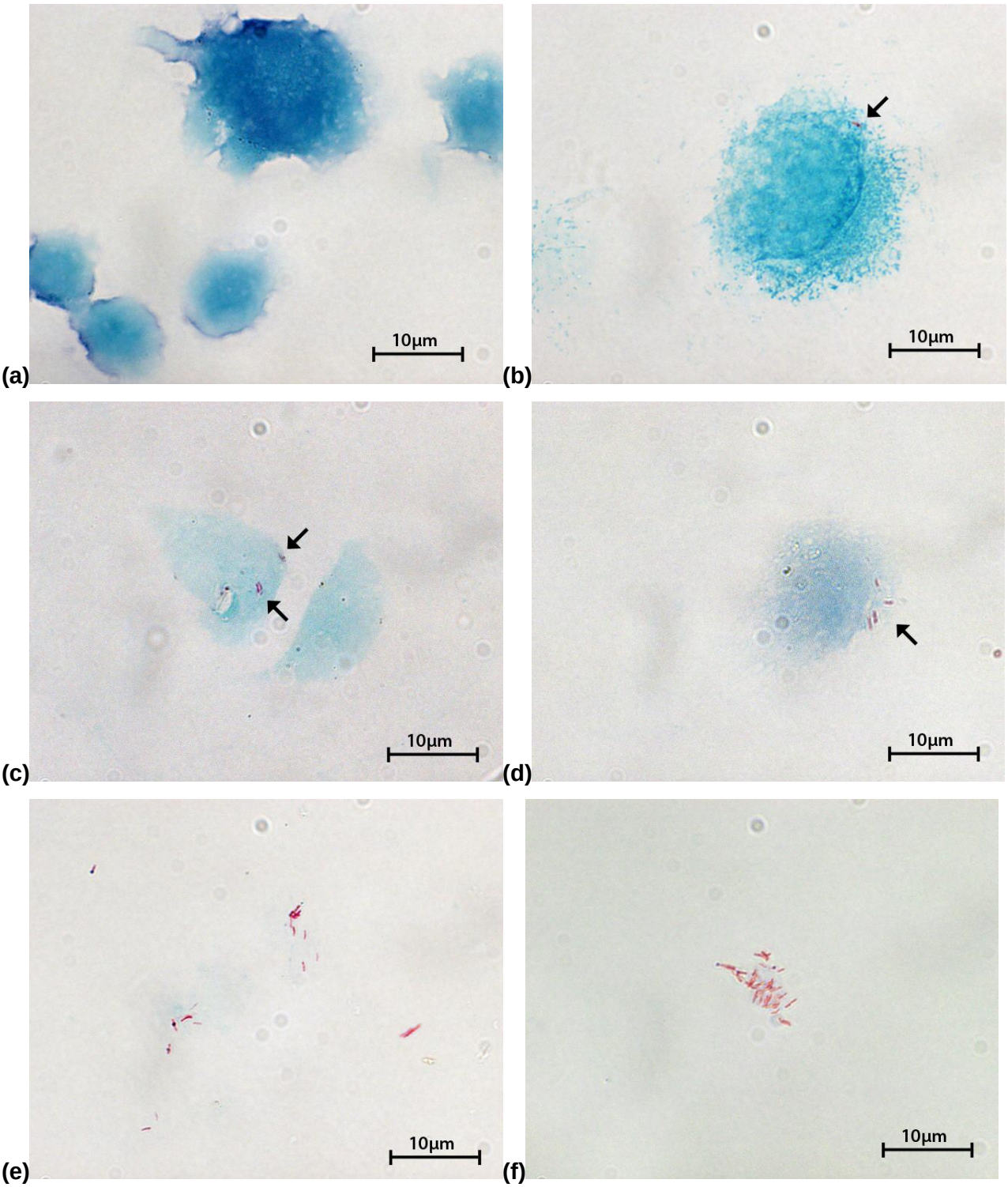



**Fig. 5.6. Micrographs of ZN stained cells infected with *M. tb***

**(a) MOI of 0**

**(b) MOI of 1**

**(c) and (d) MOI of 3**

**(e) and (f) MOI of 5**

Macrophages were infected with increasing amounts of bacilli, at ratios of one, three and five bacilli or CFUs per cell, corresponding to a MOI of 1, 3 and 5. A control of uninfected macrophages was also included. The micrographs of the ZN stained cells (Fig. 5.6.) showed that as the MOI increased, the percentages of cells infected with at least one bacillus also increased. It was observed that approximately 60% of cells with an MOI of 1 (Fig. 5.6.b.) did indeed contain one bacillus, while the remaining 40% of the cells contained no intracellular *M. tb*. At an MOI of 3 (Fig. 5.6.c and d.), 95% of cells contained three bacilli whilst the remaining 5% either contained one intracellular bacillus or more than 3 bacilli per macrophage. With an MOI of 5 (Fig. 5.6.e. and f.), cell debris and many free bacilli were present, indicating an overload of bacilli resulting in macrophage apoptosis. It was thus decided to use a MOI of 3 for the intracellular assays as the majority of cells were viable and successfully infected with at least three bacilli per cell.

### *5.3.4. Intracellular antimycobacterial activity of test samples*

The intracellular antimycobacterial activity of the tested samples is represented as the percentage of intracellular *M. tb* growth inhibition (Fig. 5.7.) as compared to the growth or number of *M. tb* CFUs present for the growth control (untreated infected macrophages) which was expressed as 100% growth (or rather 0% inhibition). The number of enumerated CFU's for the growth control ($1.02 \times 10^5$ CFU/mL) corresponded well with the amount of bacilli used to infect the cells ($3 \times 10^5$ CFU/mL).

None of the samples tested at concentrations below their antimycobacterial MIC exhibited inhibition of intracellular *M. tb* growth, except for INH, which still exhibited *M. tb* inhibition of approximately 70% at a concentration of 0.10 µg/mL (half the original exctracellular MIC value). The only samples that exhibited any noteworthy inhibition of *M. tb* growth were INH with 98% inhibition at 0.20 µg/mL, *K. vesicatoria* with 80% inhibition at 50.0 µg/mL and *E. natalensis* with 60% inhibition of intracellular *M. tb* growth at 8.00 µg/mL. Samples that were not active intracellularly even at their extracellular antimycobacterial concentrations included C3 (synergistic combination 3) and the pure compound 7-MJ. The inactivity of 7-MJ is surprising as this is a fairly fat soluble compound that would readily cross the cell membrane. Another reason for sample inactivity could be due to a pH change inside of the macrophages where the lysosomes containing the *M. tb* has a much lower pH (acidic) as the rest of the cell, which could have a drastic effect on the structure and activity of the compounds. The highest percentage of DMSO (0.25%) present in the test samples did not affect *M. tb* growth as no inhibition was evident with the solvent control. Compound 1 and *P. sidoides* were not included due to their MIC concentrations being above $IC_{50}$ values. Compound 2A and the isomer mixture were not included due to low antimycobacterial activity and low volumes of compound.

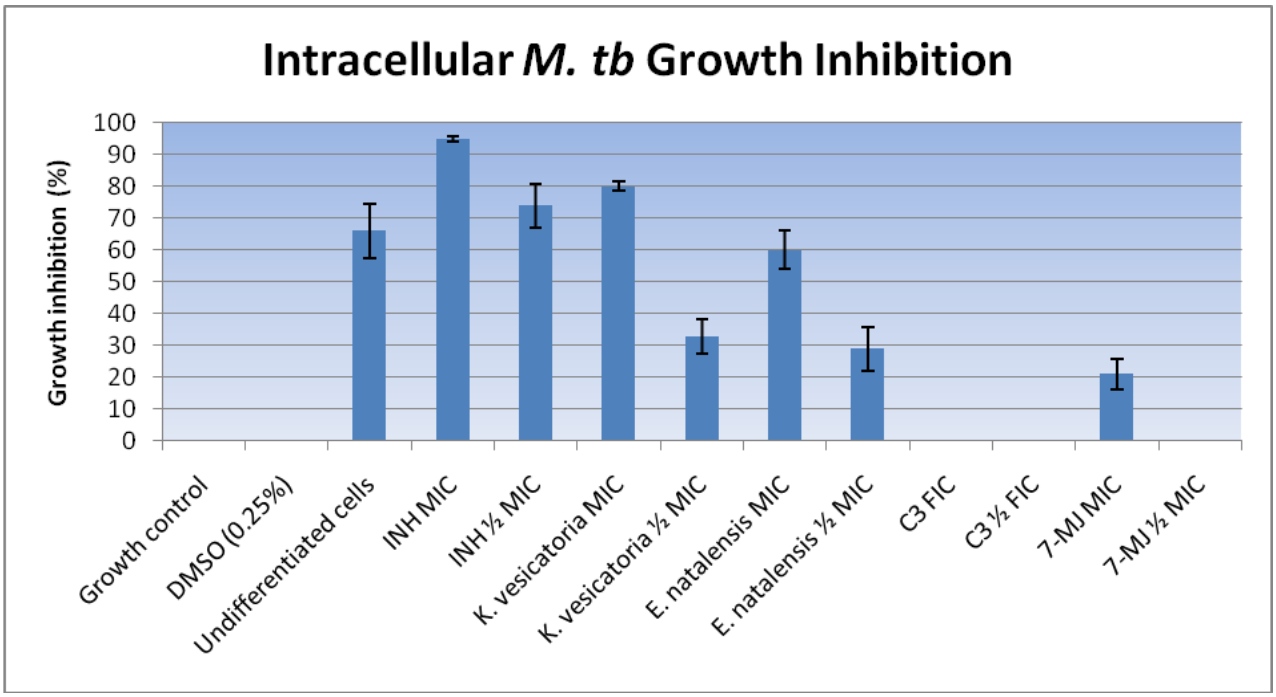



**Fig. 5.7. Intracellular *M. tb* growth inhibition by test samples**

**Table. 5.3. Intracellular antimycobacterial activity of test samples**

| Sample | Growth ± SD[a] (%) | Inhibition (%) |
|---|---|---|
| Growth control [b] | 100 ± 18.21 | No inhibition |
| DMSO (0.25%) | 100 ± 23.74 | No inhibition |
| Undifferentiated cells | 34.0 ± 8.623 | 66.0 |
| **INH:** | | |
| MIC (0.20 µg/mL) | 5.00 ± 0.809 | 95.0 |
| ½ MIC (0.10 µg/mL) | 26.0 ± 6.853 | 74.0 |
| ***K. vesicatoria*:** | | |
| MIC (50.0 µg/mL) | 20.0 ± 1.413 | 80.0 |
| ½ MIC (25.0 µg/mL) | 67.0 ± 5.374 | 33.0 |
| ***E. natalensis*** | | |
| MIC (8.00 µg/mL) | 40.0 ± 6.098 | 60.0 |
| ½ MIC (4.00 µg/mL) | 71.0 ± 6.915 | 29.0 |
| **Combination 3 (C3)** | | |
| FIC (6.28 µg/mL) | 312 ± 58.85 | No inhibition |
| ½ FIC (3.14 µg/mL) | 516 ± 39.05 | No inhibition |
| **7-MJ** | | |
| MIC ( 0.50 µg/mL) | 79 ± 4.849 | 21.0 |
| ½ MIC (0.25 µg/mL) | 337 ± 15.29 | No inhibition |

[a] *M. tb* growth expressed as a percentage of the mean CFU/mL compared to the growth control

[b] Mean of growth control equal to 1.02 x $10^5$ CFU/mL

Undifferentiated cells bound to approximately 34% of *M. tb* bacilli when CFUs where compared to the growth control. It was suspected that the undifferentiated cells would not bind to any of the bacilli, but seeing that *M. tb* bacilli were prepared in 10% FCS medium, as proposed by Passmore *et al*. (2001), some of the monocytes could have been primed to phagocytose the opsonized bacilli. The possibility of extracellular bacilli still being present after the initial washing of cells following the infection period and the substitution of medium three times, before the cells were lysed, is highly unlikely.

### *5.3.5. Cytokine detection via Cytometric bead array immunoassay (CBA)*

This microparticle-based flow cytometric immunoassay has been proven in comparison studies to have comparable analytical sensitivity to conventional ELISAs (Pandey *et al*., 2010). The levels (pg/mL) of the six cytokines (IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α) were successfully acquired and analysed. The measurement of IL-12 was unsuccessful due to faults with the purchased Flex set. Initially, supernatants from cells 24hrs after sample treatment were used for the CBA assay. Measurement of the 24hr supernatants was unsuccessful due to technical difficulties with the BD FACSArray bioanalyzer, consequently samples were lost. Acquisition was repeated with supernatants from infected and uninfected cells treated with samples after 72 hrs (three days). It was suspected that the cytokine levels from Day 3 would be much lower than what would have been expected after only 24hrs as cytokines have fast and shortlived effects. However, cytotoxicity was measured 72hrs after sample addition and can thus be correlated with the cytokine results.

Considering the experimental outcomes for the uninfected macrophages (Table. 5.4.), none of the test samples produced any toxic effects, as most samples exhibited similar cytokine profiles to that of the untreated differentiated cells. Also, none of the test samples produced TNF (a tell-tale sign of toxicity), except for very low levels (2.52 pg/mL) produced by *K. vesicatoria* at 50 µg/mL which was the highest concentration tested for this extract. To measure the effect of the 2.5% PANTA, present for all samples, a control of differentiated cells without PANTA was included. Similar cytokine production was seen for the differentiated cells without PANTA as for untreated cells with PANTA. The highest concentration of DMSO present in the assay (0.25%) seemed to neutralise any cytokine production.

Macrophages are the first line of defense against *M. tb*. In response to infection with mycobacteria, macrophages produce cytokines including the Th1-type cytokine, IL-12. These cytokines synergistically stimulate production of IFN-γ by CD4+ T cells and NK cells. IFN-γ ligand binds to its receptor on the macrophage cell surface and activates the macrophage resulting in enhanced TNF-α production, production of other cytokines and chemokines, upregulation of MHC class II expression, enhanced antigen processing, and production of reactive oxygen species (Dorman and Holland, 2000). Cytokines from macrophages can thus have an effect on CD4+ cells and can

modulate the eventual Th1 or Th2 response to a *M. tb* infection, according to the cytokines produced.

**Table. 5.4. Cytokine profiles (pg/mL) of sample treated macrophages (72hr treatment)**

| Test Sample | Th1 Cytokines | | | Th2 Cytokines | | | Response |
|---|---|---|---|---|---|---|---|
| | IL-2 | IFN-γ | TNF | IL-4 | IL-5 | IL-10 | (Th1/Th2) |
| Differentiated (+P) [b] | 0.00 | 0.00 | 0.00 | 0.00 | 1.31 | Er [c] | Th2 |
| Differentiated (-P) [d] | 0.00 | 0.00 | 0.00 | 0.00 | 1.17 | 1.32 | Th2 |
| DMSO (0.25%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| **INH:** | | | | | | | |
| ½ MIC (0.10 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| MIC (0.20 µg/mL) | 0.00 | 0.00 | 0.00 | 1.85 | 0.00 | 1.39 | Th2 |
| $IC_{50}$ (200 µg/mL) | 0.00 | 0.00 | 0.00 | Er | 0.00 | 2.17 | Th2 |
| ***K. vesicatoria:*** | | | | | | | |
| ½ MIC (25.0 µg/mL) | 0.00 | 2.87 | 0.00 | 4.19 | 1.44 | 3.02 | Th2 |
| MIC (50.0 µg/mL) | 0.00 | 5.61 | 2.52 | 5.15 | 1.96 | 3.51 | Th2 |
| $IC_{50}$ (41.3 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| ***E. natalensis*:** | | | | | | | |
| ½ MIC (4.00 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 1.26 | 1.98 | Th2 |
| MIC (8.00 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| $IC_{50}$ (12.22 µg/mL) | 0.00 | 3.85 | 0.00 | 2.72 | 1.26 | 2.64 | Th2 |
| **Combination 3** | | | | | | | |
| ½ FIC (6.28 µg/mL) | 0.00 | 0.00 | 0.00 | Er | 1.17 | 1.98 | Th2 |
| FIC (3.14 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| $IC_{50}$ (121 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| **7-MJ:** | | | | | | | - |
| ½ MIC (0.25 µg/mL) | 0.00 | 2.87 | 0.00 | 2.46 | 1.26 | 2.59 | Th2 |
| MIC (0.50 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.57 | Th2 |
| $IC_{50}$ (18.0 µg/mL) [e] | 0.00 | 4.84 | 0.00 | 2.02 | 1.59 | 2.97 | Th2 |
| ***P. sidoides*** $IC_{50}$ (43.0 µg/mL) | 0.00 | 3.12 | 0.00 | 1.43 | 1.04 | 2.83 | Th2 |
| **Compound 1** $IC_{50}$ (16.4 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.73 | Th2 |

[a] Undifferentiated U937 cells with 2.5% PANTA [b] Differentiated U937 cells with 2.5% PANTA

[c] Error: cytokine levels were out of range and could not be acquired

[d] Differentiated U937 cells without 2.5% PANTA

[e] Fifty percent inhibitory concentration ($IC_{50}$) of 7-MJ on the THP monocytic cell-line (???).

Macrophages might play a significant role in providing the microenvironment required for optimal function of Th1 effectors at the site of infection (Jankovic *et al*., 2001). None of the samples stimulated the production of the Th1 cytokine, IL-2 and very few of the samples produced any of the remaining two Th1 cytokines, IFN-γ and TNF (Table. 5.4.). The highest level seen for IFN-γ was for *K. vesicatoria* (5.61 pg/mL) at 50.0 µg/mL, followed by 7-MJ (4.84 pg/mL) at 18.00 µg/mL, *E. natalensis* (3.85 pg/mL) at 12.22 µg/mL and *P. sidoides* (3.12 pg/mL) at 43.0 µg/mL. More samples produced the Th2-type cytokines (IL,-4,-5 and -10), albeit at low levels, the highest being 3.51 pg/mL of IL-10 produced by the stimulation of *K. vesicatoria* (50.0 µg/mL). Untreated cells appear to be in a Th2-cytokine producing state, indicating that cells could have been in this state even before sample treatment. Very low levels of Th2 cytokines (especially IL-4 and IL-10) are needed to negate the effects of Th1 cytokines, even if the latter are at higher concentrations (Ghadimi *et al*., 2010). In addition, the Th1-type cytokine IFN-γ is synthesized earlier than the type 2 cytokine, IL-4 (Breytenbach *et al*., 2001). The Th2 polarised state of the cells could be the reason for samples producing more Th2 type cytokines.

Sample treated cells, infected with *M. tb* (Table. 5.5.), had very similar cytokine profiles when compared to the uninfected cells (Table. 5.5.). Differences were seen for the control cells which produced no cytokines, except for the differentiated cells containing PANTA, which expressed an IL-10 level of 1.88 pg/mL. Compared to the uninfected cells, where *E. natalensis* stimulated the production of IFN-γ, IL-4, -5 and -10, the infected cells produced only IL-10, at a lower level of 1.73 pg/mL compared to 2.64 pg/mL. Again, the most varied and peak levels of cytokine production were seen for the highest concentrations of *K. vesicatoria* (50.0 µg/mL), 7-MJ (18.00 µg/mL) and *P. sidoides* (43.0 µg/mL). The most striking result was observed for infected cells treated with 7-MJ. All the cytokines tested for were produced at high levels (relative to other samples). No other sample stimulated the production of IL-2, which was at 7.64 pg/mL for 7-MJ treated cells. The remaining two Th1-type cytokines, IFN-γ and TNF-α, were produced at 17.31 and 8.58 pg/mL, respectively. The Th2-type cytokines, IL-4, -5 and -10 were produced at levels of 15.52, 4.04 and 8.51 pg/mL, putting the Th1-cytokines at a slightly higher level. *Pelargonium sidoides* treated cells also indicated a cytokine profile leaning more towards Th1 than Th2 subsets. It was quite surprising that Compound 1 did not have more of an effect on cytokine production as plant sterols are known to have immunomodulating effects (Breytenbach *et al*., 2001 and Bouic, 2002). It is interesting to note that a correlation can be made between the intracellular *M. tb* growth inhibition of *K. vesicatoria* (80%) and the cytokine stimulation of this sample at 50.0 µg/mL. The antimycobacterial activity of the extract itself and the presence of IFN-γ, which activates macrophage killing of intracellular organisms, could account for the inhibition of *M. tb* intracellularly.

**Table. 5.5. Cytokine profiles of *M. tb* infected sample treated macrophages (72hr treatment)**

| Test Sample | Th1 Cytokines | | | Th2 Cytokines | | | Response |
|---|---|---|---|---|---|---|---|
| | IL-2 | IFN-γ | TNF | IL-4 | IL-5 | IL-10 | (Th1/Th2) |
| Differentiated (+P) [b] | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.88 | Th2 |
| Differentiated (-P) [c] | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| DMSO | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| **INH:** | | | | | | | |
| ½ MIC (0.10 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | Th2 |
| MIC (0.20 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 1.11 | 2.03 | Th2 |
| $IC_{50}$ (200 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.39 | Th2 |
| ***K. vesicatoria:*** | | | | | | | |
| ½ MIC (25.0 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| MIC (50.0 µg/mL) | 0.00 | 5.10 | 2.68 | 4.67 | 1.91 | 3.51 | Th2 |
| $IC_{50}$ (41.3 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| ***E. natalensis*:** | | | | | | | |
| ½ MIC (4.00 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| MIC (8.00 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.57 | Th2 |
| $IC_{50}$ (12.2 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.73 | Th2 |
| **Combination 3** | | | | | | | |
| ½ FIC (6.28 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| FIC (3.14 µg/mL) | 0.00 | 0.00 | 0.00 | 1.43 | 0.00 | 1.32 | Th2 |
| $IC_{50}$ (121 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| **7-MJ:** | | | | | | | |
| ½ MIC (0.50 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| MIC (0.25 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| $IC_{50}$ (18.0 µg/mL) [d] | 7.64 | 17.31 | 8.58 | 15.52 | 4.04 | 8.51 | Th1 |
| ***P. sidoides*** $IC_{50}$ (43.0 µg/mL) | 0.00 | 3.24 | 2.12 | 0.00 | 1.33 | 3.21 | Th1 |
| **Compound 1** $IC_{50}$ (16.4 µg/mL) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |

[a] Undifferentiated U937 cells with 2.5% PANTA

[b] Differentiated U937 cells with 2.5% PANTA

[c] Differentiated U937 cells without 2.5% PANTA

[d] Fifty percent inhibitory concentration ($IC_{50}$) of 7-MJ on the myelomonocytic cell line THP-1 (???).

## 5.4. Conclusion

For the purpose of determining intracellular antimycobacterial activity of samples and the subsequent measurement of Th1- and Th2-type cytokines (including IFN-γ) phorbol esters were used to induce differentiation of U937 monocytes to activated macrophages. According to

macrophage morphology criteria, PMA was found to be the preferred molecule for cell differentiation after only 24hrs of stimulation with a concentration of 0.10 µg/mL.

Even when conducting *in vitro* tests, it is important to understand the margin of safety that exists between an extract or compound dose needed for the desired effect, and the dose that produces unwanted side effects. Test samples were evaluated for their cytotoxicity on differentiated U937 cells to eliminate those samples with an antimycobacterial concentration above their cytotoxic concentration. The only samples that could not be tested at their antimycobacterial concentration was the tannin free extract of *K. vesicatoria*, the crude ethanol extract of *P. sidoides*, Compound 1 and Compound 2A. The tannin-free extract of *K. vesicatoria* was moderately less toxic to the macrophages than the crude *K. vesicatoria* extract, with an $IC_{50}$ of 64.71 µg/mL compared to 41.25 µg/mL. Interestingly, the drug combination of C3 was two-fold less toxic than its *K. vesicatoria* component but twice as cytotoxic as its INH component.

A ratio of three *M. tb* bacilli per macrophage cell was determined as the optimal MOI. Approximately 90% of the macrophages were successfully infected and the majority of cells were viable. Previous attempts at determining intracellular antimycobacterial activity according to methods described by Rastogi *et al*. (1991) and Mativandlela (2009) have been unsuccessful largely due to an overload of bacilli and contamination with organisms other than *M. tb*. Determining the correct ratio of infectious agents per phagocytic cell and the addition of the antimicrobial mixture, PANTA (2.5%), effectively overcame these pitfalls. The only samples that exhibited any significant intracellular antimycobacterial activity were the crude extracts of *K. vesicatoria* and *E. natalensis* with 80 and 60% *M. tb* growth inhibition at concentrations of 50.00 and 8.00 µg/mL, respectively.

Overall, the levels of cytokine production was very low, most likely due to the experimental circumstances where the supernatant was initially collected at 24hrs after incubation and replaced with new sample-containing medium and only collected again after another 48hrs. The production of cytokines from these cells thus gives an idea of long-term cytokine production in response to the test samples. Treatment with samples slightly altered the production of both type 1 and 2 cytokines leaning more towards Th2 subsets, but did not induce a marked polarized type 1 to type 2 state. A slight Th1-type response was seen for infected cells treated with 7-MJ and *P. sidoides* at their $IC_{50}$ values of 18.0 µg/mL and 43.0 µg/mL. Reflecting back at experimental procedure, it would have been sensible to also include the $IC_{50}$ values of all the test samples for CFU enumeration to be able to accurately compare cytokine profiles with intracellular *M. tb* inhibition.

It is increasingly difficult to interpret the experimental outcome of cytokines secreted by macrophages as so many different theories have been proposed (Condos, 2007; Denis, 1991;

Flynn *et al*., 1993 and 1995; Ghadimi *et al*., 2010; Grange and Stanford, 1995; Kaufmann and Schaible, 2003; Mosmann and Coffman, 1989; Romagnani, 1994). An *in vitro* test, such as performed in this study, can give an idea as to what is happening at a single cell level. Part of the difficulty when interpreting experimental outcomes based on the effects of samples on macrophages is that they may not reflect accurately the behaviour of the same cytokines in a more complex cytokine environment, such as the numerous interacting cells present in the human host. An infection is likely to induce a complex cytokine profile, including both stimulatory and inhibitory cytokines. Consequently, multiple cytokine effects on macrophages are exerted simultaneously and the net result may be ultimately determined by both the nature of cytokines present and their concentration levels.

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# CHAPTER 6
# GENERAL DISCUSSION AND CONCLUSION

## 6.1. General discussion and conclusion

Proper tuberculosis care and control averted up to 6 million deaths and cured 36 million people between 1995 and 2008 (Lönnroth *et al*., 2010). However, this disease is still causing considerable burden and loss of productivity. Global control of tuberculosis is far from complete. There were 9.4 million estimated new cases of tuberculosis in 2009 and HIV continues to fuel the epidemic by providing a large reservoir of highly susceptible individuals, especially in Africa. With 1. 8 million estimated deaths every year, tuberculosis still takes a huge toll, especially for the poorest people. It is a leading cause of death in people in the most economically productive age-groups. The direct and indirect costs of tuberculosis, and the social consequences, are often catastrophic for the individual patient, the family, and the wider community. A number of efficacious anti-tubercular agents were discovered in the late 1940s and 1950s with the last, rifampicin, introduced in the 1960s (Schraufnagel, 1999). These agents had reasonable efficacy and, when used in combination, would preclude the development of drug resistance. The use or (in most cases) misuse of these drugs over the years has led to an increasing prevalence of multiple- and extensively-drug resistant (M- and XDR) strains, establishing an urgent need to develop new effective agents (Cox *et al*., 2003; Nachega and Chaisson, 2003). There have been a number of practical obstacles to the development of new anti-TB agents, among them a lack of economic incentive due to the predominance of disease in the developing world. In spite of the difficulties, there has recently been a renewed interest in finding new agents as well as the development of a roadmap for their development (Global Allicance for TB Drug Development, 2001).

As outlined in Chapter 2, the challenge of discovering new, urgently needed anti-TB drugs from natural sources requires truly interdisciplinary research. Mycobacteriology and innovative natural products have to be developed and employed together in order to meet demands for effective natural anti-TB treatments.

During the present study, fractions obtained from the tannin clean-up of the *K. vesicatoria* ethanol extract, exhibited no significant antimycobacterial activity when tested against two mycobacteria, *M. smegmatis* and *M. tuberculosis.* The highest activity of these fractions was for the hexane fraction while the tannin free fraction had poor antimycobacterial activity. The activity of the whole *Knowltonia* extract remained superior. It is thus clear that tannins do not account for the antimycobacterial activity of the aerial ethanol extract of *K. vesicatoria*. The most likely scenario is that non-polar components synergistically interact with polar components to exert the eventual

inhibitory activity seen for the whole extract. In future, combining permutations of the different partitioned extracts and testing these combinations for antimycobacterial activity is recommended.

The antimycobacterial results obtained for the *K*. *vesicatoria*, *P. sidoides* and *E. natalensis* extracts against *M*. *tuberculosis* using the BACTEC radiometric method and an INT colorimetric broth dilution test, showed corresponding results with previous MIC values for these extracts. Pronounced synergistic antimycobacterial activity was exhibited by the combination of *K. vesicatoria* with the antitubercular drug INH. Due to the inhibitory activity of *K. vesicatoria* extract against two INH resistant strains of *M. tb,* it is proposed that the *Knowltonia* extract has a very different mechanism of action towards inhibiting *M. tb* growth as compared to INH. The direct colorimetric microplate dilution assay employing INT as the growth indicating agent, proved to be a suitable procedure for screening new antimycobacterial compounds against *M. tuberculosis*. Further standardisation of the test is necessary and should include susceptibility testing of all the current first-line antitubercular drugs before the assay is to be used as a full scale test for novel drug susceptibility testing.

Bioassay-guided fractionation is the process that is currently used to identify the active principle(s) contained in crude natural products preparations (e.g., extracts). Due to technological advancements in chromatography and spectroscopy (e.g. NMR) over the past two decades, the sensitivity of natural product fractionation procedures has increased dramatically. This sensitivity opens new alleys not only for unstudied materials, but also for previously investigated genera, and provides access to unexpected chemical types and novel compounds (Phillipson, 1995). Through bioassay-guided fractionation and isolation procedures stigmasta-5,23-dien-3-ol was isolated as a moderately active *in vitro* antimycobacterial component of *K. vesicatoria*. The lactone *5-(hydroxymethyl)furan-2(5*H*)-one*, exhibited low inhibitory activity of *M. tb*. No previous antimycobacterial activity has been reported for any of these two compounds. Together with the inactivity seen for fractions obtained from the tannin clean-up of *K. vesicatoria*, and the moderate activity of the isolated compounds against *M. tb*, it is reasonable to attribute this plant's antimycobacterial activity to synergistic interactions of known and unknown components found within the crude aerial ethanol extract.

Mycobacterial enzymes involved in the maintenance of Mycothiol, such as Mtr, could make appealing drug targets and another possibility is to reduce the effect of endogenous mycothiol to inhibit enzymes involved in degrading the mycothiol-antibiotic complex. Since there is no exact mammalian equivalent to the mycothiol pathway it should be possible to achieve selective inhibition of the mycothiol biosynthesis. The investigation on the NADPH oxidase activity of Gtr, found that *K. vesicatoria* failed to exhibit any NADPH oxidase activity. Evidently, Gtr and presumably Mtr, is not the target for the antitubercular activity of this extract. The isolated lactone,

(*5-(hydroxymethyl)furan-2(5*H*)-one*) and the crude ethanol extract of *P. sidoides* exhibited pronounced inhibition of Gtr. Slight subversive substrate activity was seen for the crude chloroform extract of *E. natalensis*. Stigmasta-5,23-dien-3-ol exhibited reversible inhibition of Gtr. Further investigations such as enzyme concentration- and time- dependant assays should be carried out for the crude extract of *E. natalensis* and both the isolated compounds (*5-(hydroxymethyl)furan-2(5*H*)-one*) and Stigmasta-5,23-dien-3-ol on both Gtr and Mtr to establish selective subversive substrate and inhibitory properties.

For the purpose of determining intracellular antimycobacterial activity of samples and the subsequent measurement of Th1- and Th2-type cytokines, PMA was found to be the preferred molecule to induce differentiation of U937 monocytes to activated macrophages. Macrophage cytotoxicity of the tannin-free extract of *K. vesicatoria* was less pronounced than the crude *K. vesicatoria* extract. Interestingly, the drug combination of C3 was two-fold less toxic than its *K. vesicatoria* component but twice more cytotoxic than its INH component. A ratio of three *M. tb* bacilli per macrophage cell was determined as the optimal MOI for infecting macrophages. The only samples that exhibited any significant intracellular antimycobacterial activity were the crude extracts of *K. vesicatoria* and *E. natalensis*.

Recognition of the roles of cytokines, such as IFN-γ, in human host defense against intracellular pathogens emphasizes the importance of research to understand the mechanisms by which IFN-γ activates macrophage killing of intracellular organisms, and the mechanisms by which pathogens such as *M. tuberculosis* apparently circumvent macrophage killing. Better understanding these mechanisms will lead to the development of rational preventive and therapeutic strategies directed against *M. tuberculosis* and other intracellular pathogens. Treatment with samples slightly altered the production of both type 1 and 2 cytokines leaning more towards Th2 subsets, but did not induce a marked polarized type 1 to type 2 state. A slight Th1-type response was seen for infected cells treated with 7-MJ and *P. sidoides*.

A herbal formulation or plant compounds that can induce the Th1 response and subsequently suppress the Th2 response by increasing or decreasing the related cytokines, can be a valuable adjuvant to current anti-TB treatments. Further studies are required to confirm the immunological activity of *K. vesicatoria,* 7-MJ and *P. sidoides* by evaluating various other cytokines such as IL-12. To determine if cytokines from macrophages would have an effect on CD4+ cells towards modulating the eventual Th1 or Th2 response to a *M. tb* infection, the next step would be to carry out co-cultures of infected macrophages with T-helper cells or better yet, *in vivo* cytokine profiles as described by Breyetenbach *et al*. (2001) that may better reflect the net effect of cytokine responses.

Drug therapy of mycobacterial infections occurs against the backdrop of host immunity and is of diminished merit without it. There is a need to develop second-line therapeutic agents, both natural and synthetic, in view of the twin problems of resistance and persistence. Such therapeutic agents may have antimycobacterial potential or may function as immunomodulators, thereby enhancing the immune status of the affected host, enabling it to combat the disease better. An important reason for the current failure to control TB is that, even when applying the best available chemotherapy, treatment must be continued for at least six months. This treatment regimen is not a realistic option in limited-resource countries such as South-Africa, or even in large cities of developed countries, because after a few weeks of treatment the patients start to feel well again and stop taking the drugs. There are two main reasons for prescribing such long-term treatments. The first is that the antibiotics kill the vast majority of the bacilli within a few days, but persisting bacteria are not killed by the drugs. These persisting bacilli may be in a true stationary phase with very low metabolism, and may be non-replicating or replicating very slowly (latent infection). The other reason is the necrotizing tissue response that is analogous to the Koch phenomenon. Still today, the task for the researchers working in this field is to understand the differences between protective immunity and progressive disease, including the Koch phenomenon. The final aim is to learn how to replace the pathological immune response (Th2 response) by the protective one (Th1 response), and with such knowledge, to design short-course chemotherapy schemes supplemented with immunotherapy or even natural plant derived antimycobacterial and immunomodulatory compounds, which would enable TB control worldwide, especially in developing countries.

The various aspects of the collaborative challenge faced in TB drug discovery are applicable to other infectious agents. It is the interplay of co-developed innovative methods on both the biological and the natural products chemical perspectives that will improve the chances of success.

## 6.2. References

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