Isolation and characterization of the cytotoxicity, intracellular bioactivity and mechanism of antimycobacterial action of *Euclea natalensis*-derived naphthoquinones

By

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SUMMARY

The major cause of HIV-related mortality in Sub-Saharan countries is pulmonary tuberculosis (TB), which is an escalating threat due to the emergence of multidrug resistant (MDR) and extremely multidrug resistant (XDR) TB. There is clearly an urgent requirement for the identification of novel, affordable anti-TB (as well as anti-HIV) drugs.

This study was undertaken with the objective of isolating and characterizing the anti-mycobacterial potential of 3 naphthoquinones, i.e. neodiospyrin, diospyrin and 7-methyljuglone present in the roots of *Euclea natalensis.* The laboratory research included: i) isolation of diospyrin and neodiospyrin, from the roots of *E. natalensis*; ii) assessment of the cytotoxicity of these agents and synthetic 7-methyljuglone for eukaryotic cells (Vero and THP-1 cells); iii) determination of the intracellular bioactivities of the naphthoquinones against the H37Rv strain of *Mycobacterium tuberculosis* (MTB); and iv) mechanistic studies designed to investigate the effects of the test agents on cation (K^+/ Ca^{2+}) fluxes and energy metabolism (ATP levels) in MTB and *M. smegmatis.*

With respect to the first objective, the naphthoquinones (diospyrin and neodiospyrin) were isolated from crude methanol extracts of crushed roots using chromatography and spectroscopic analysis. The yields of the compounds were 0.16 %, 0.32 %, and 0.12 %

for neodiospyrin, diospyrin (isolated from plant) and synthetic 7-methyljuglone (synthesised in laboratory), respectively.

The effects of the compounds (0.3-50µg/ml) on the viability of Vero and THP-1 cells were measured using the XTT assay (sodium 3'-[1-(phenyl amino-carbonyl)-3, 4 tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) based on cellular metabolic activity. All 3 test compounds were found to possess cytotoxic activity at 1.5-12.5µg/ml) for both cell lines.

Intracellular bioactivity of the test agents was measured using MTB-infected THP-1 cells as a surrogate for infected human macrophages. Following exposure of the MTB-infected cells to the test naphthoquinones, at a concentration range of 6.25-25µg/ml, for 5 days, the cells were lysed and the viability of MTB in the lysates was then measured using the BACTEC radiometric system. All 3 test agents were found to be bioactive intracellularly, with complete inhibition of growth detected at 12.5, 25, and 6.25µg/ml in the case of neodiospyrin, diospyrin, and synthetic 7-methyljuglone respectively.

The effects of the 3 naphthoquinones on mycobacterial cation fluxes were measured according to the magnitude of uptake of $^{86}Rb^{+}$ (a surrogate for K^{+}) and $^{45}Ca^{2+}$, while ATP was measured using a chemiluminescence procedure. None of the test agents was found to affect Ca^{2+} uptake by the bacteria. However, all 3 test agents were found to be potent inhibitors of uptake of K^{+} by MTB and *M. smegmatis*, with inhibition detected at sub-microgram concentrations of these agents. All 3 test agents, especially synthetic 7-

methyljuglone, were found to interfere with energy metabolism in MTB, manifested as decreases in mycobacterial ATP levels.

Synthetic 7-methyljuglone which has the lowest MIC value for MTB (0.5µg/ml), and which was the most potent inhibitor of energy metabolism in MTB, shows promise as a potential anti-TB agent.These agents also are of potential value in drug modelling, possibly in the design of novel anti-TB agents which selectively target mycobacterial K^+ transporters.

Key Words: naphthoquinones, diospyrin, neodiospyrin, 7-methyljuglone, cytotoxicity intracellular bioactivity, potassium transport, ATP levels.

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LIST OF ABBREVIATIONS

AIDS-Acquired Immune Deficiency Syndrome

ATP-Adenosine triphosphate

BCG-Bacillus Calmette Guerin

BSA-Bovine serum albumin

CFU-Colony forming units

DMSO-Dimethyl sulfoxide

DNA-deoxyribonucleic acid

EMB-Ethambutol

FCS-Fetal calf serum

GI-Growth Index

H' NMR- Hydrogen-1 Nuclear magnetic resonance

HIV-Human Immunodefiency Virus

HPLC-High Performance Liquid Chromatography

IFN-γ-Interferon-γ

IL-Interleukin

INH-Isonicotinic acid hydrazine

KONO-Potassium free Sodium free

LAM-Lipoarabinomannan

MCP-1-Macrophage chemoacttractant protein-1

MDR-TB-Multidrug Resistant Tuberculosis

MEM-Minimum essential medium

MHC II-Major histocompatibilty class II

MIC-Minimum Inhibitory Concentration

MIP-1α-Macrophage inflamatory protein 1-alpha

MRC-Medical Research Council

MTB-*Mycobacterium tuberculosis*

MTT-[3-(4.5-dimethylthiazol-2yl)-2.5-diphenyl tetrazolium bromide]

NK-Natural killer

NMR-Nuclear Magnetic resonance

OADC-Oleic albumin dextrose catalase

OD-Optical Density

PBS-Phosphate-Buffered Saline

PMA- Phorbol 12-Myristate 13-Acetate

PZA-Pyrazinamide

Rb-Rubidium

RMP-Rifampin

SDS-Sodium dodecyl sulfate

SP-A-Surfactant protein-A

SYN-MJ-Synthetic 7-methyjuglone

TB-Tuberculosis

Th $CD4^+$-T-helper lymphocytes cluster of differentiation 4^+

TLC-Thin Layer chromatogarphy

TLR-2-Toll-like receptor-2

TNF-Tumor necrosis factor

UV-Ultraviolet

WHO-World Health Organisation

XDR-TB-Extensively/extremely Drug Resisitant Tuberculosis

XTT-sodium 3'-[1-(phenyl amino-carbonyl)-3,4 tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate

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1.1 Introduction

Tuberculosis (TB) places a severe burden on the public health systems of developing countries with this disease being the leading infectious cause of escalating morbidity rates. Years of extensive research into effective therapeutic strategies to combat this disease have not succeeded in eradicating the causative agent, *Mycobacterium tuberculosis* (MTB). Poor drug compliance, poverty and Human Immunodeficency Virus (HIV) co-infection have contributed to the emergence of multidrug resistant strains of TB (MDR-TB).

While first line and second line antibiotics have been effective in treating MDR-TB, the world was alarmed when a new highly resistant strain of TB emerged (Lancet, 2006; Jones et al., 2007). A strain of TB was discovered in isolates of patients that was resistant to both current first and second line antibiotics, and was named as "extensively drug resistant tuberculosis" (XDR-TB). In 2006, South Africa was one of the countries hardest hit by XDR-TB when 52 of the 53 patients infected with XDR-TB died, an almost 99% mortality rate (Lancet, 2006; Koenig, 2008). The emergence of XDR-TB coupled with the increase in HIV/AIDS has intensified the need to identify new treatment strategies, especially accelerated development of antibiotics which can be active against XDR-TB in order to avoid a global public health crisis.

Globally, TB is a worldwide threat, causing 1.8 million deaths and 9.27 million new cases in 2007, making TB the predominant microbial pathogen in the world (World Health Organization Report, 2008; Barker et al., 2009).

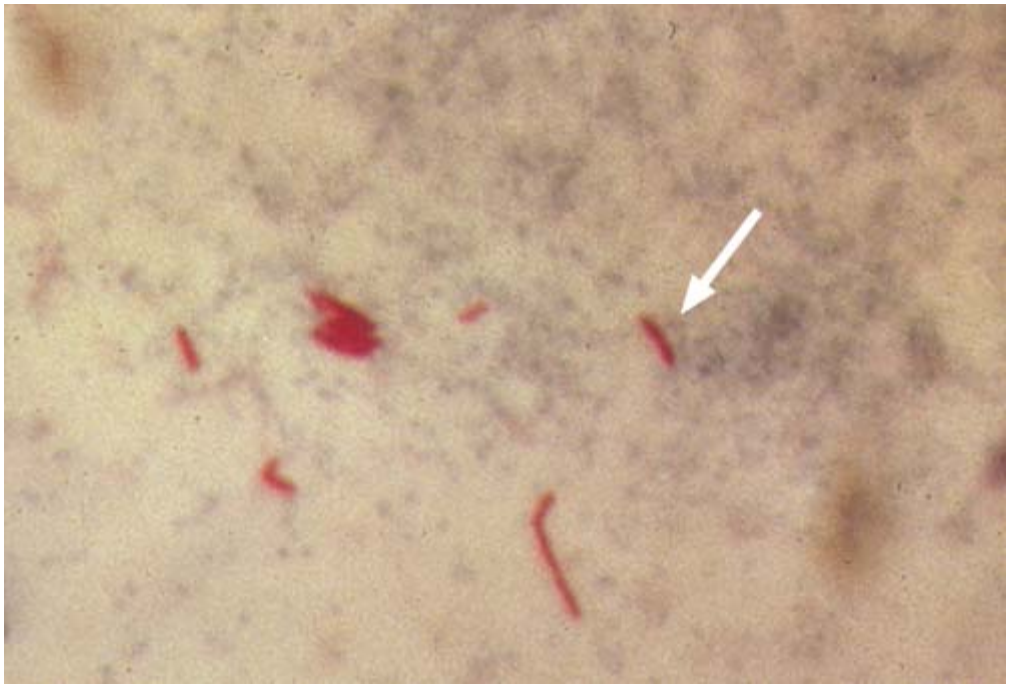


Figure 1.1 Acid-fast staining of tuberculosis bacilli as indicated by the arrow (x 100 magnification) (www.aminol-uk.com/services.htm**)**

Currently, the first-line antibiotics used in the treatment of TB are isoniazid, ethambutol, pyrazinamide and rifampin. These antibiotics are reasonably effective in treating individuals suffering from TB, but are ineffective in totally eliminating the bacilli (Ahmed et al., 2009; Karim et al., 2009). A setback of antibiotic treatment is that the course of the treatment is lengthy, usually up to six months, and involves administration of 2 to 4 specific antimycobacterial drugs often, resulting in poor compliance (Flynn and Ernst, 2000).

Poor compliance with the prescribed drug regimen coupled with improper use of antibiotics, gives rise to multidrug resistant TB (MDR-TB). Multidrug resistant

tuberculosis is defined as *in vitro* resistance of MTB to at least rifampin and isoniazid (Skenders et al., 2005; World Health Organization, 2008). The development of drug-resistance and MDR-TB is caused by chromosomal mutations in the different genes of the bacteria due to incorrect drug use. Currently, MDR-TB strains resistant to up to nine different agents have been reported. Usually, drug-susceptible TB can be cured within 6 months, but forms of TB such as MDR-TB require intensive chemotherapy for up to 2 years which is very detrimental to a patient's health, due to high levels of toxicity (Lancet, 2006). For the past few decades no new drugs have been discovered which effectively combat TB, although several are in the pipeline. (http://www.tbalert.org/resources/documents/Freire.pdf)

With the emergence of XDR-TB, pharmaceutical companies need to assign development of new anti-TB compounds as a high priority. A co-ordinated effort to screen traditional medicinal plants for anti-TB activity is a worthwhile strategy.

1.2 History of TB

It is believed that the genus *Mycobacterium* originated 150 million years ago (Hayman, 1984). The estimation of the time of origin of MTB is made possible by techniques of molecular genetics and sequencing of the genome of several species of *Mycobacterium* (Daniels, 2004). East Africa is said to be the ancestral home of the tubercle bacilli as all six circulating strains are currently found there. Skeletal abnormalities caused by tuberculosis were depicted in early Egyptian Art (Figure 1.2) (Cave, 1939; Morse et al.,

1964; Morse et al., 1967). The discovery of TB in Egyptian mummies in a DNA fragment from an Egyptian skeleton that exhibited spinal deformities is suggestive of tuberculosis vertebral involvement (Figure 1.3) (Crubezy et al., 1998).

Figure 1.2 A tomb painting at Beni Hasan showing a gardener with a localized angular deformity of the cervical-thoracic spine. (www.nanobiomed.de/_Media/fig1-6_textmedium.jpeg)

Figure 1.3 Partial destruction and curvature of the vertebral bones, caused by tuberculosis infection (www.answers.com/topic/pott-s-disease**)**

Tuberculosis was also mentioned in the Biblical books of 'Deuteronomy' and 'Leviticus' using the ancient Hebrew word 'schahepeth' (Daniel and Daniel, 1999). In India, TB has been documented as early as 3300 years ago, and in China 2300 years ago.

Tuberculosis was known in Greece as 'phthisis' (Meachen, 1936; Daniel, 1998). The father of medicine, Hippocrates, reported that phthisis attacked people between the age of eighteen and thirty five and proved to be fatal. At around the same time, the Greek physician, 'Clarrismua Galen,' wrote about tuberculosis and recommended fresh air, milk and sea voyages for treatment of this disease.

In 1865, the French military surgeon, Jean-Antoine Villemin, demonstrated the infectious nature of TB by removing an amount of purulent liquid from an individual who died from TB and then injecting it into a rabbit (Barnes, 1995). The rabbit had extensive internal TB infection; however it remained healthy (due to the unknown fact at that time that rabbits are resistant to infection with MTB).

On March 24, 1882, Herman Heinrich Robert Koch of Germany, changed the history of medicine when he demonstrated that MTB was the causative agent of TB in humans. It was discovered around the 1890s that a glycerin extract of the tubercle bacilli could be used as a remedy for tuberculosis. He isolated a substance called, 'tuberculin' from the bacilli and injected himself with 25 cm^3 of concentrated tuberculin and observed "an unusually violent attack of ague and a rapid increase of body temperature to 39.6°C" (Koch, 1891).

In 1907, Clemen Freiherr von Pirquet from Vienna showed that positive tuberculin reactions reflected latent tuberculosis (Daniel, 1998). During this era, as a result of all the information derived from clinical trials and the efforts of the above-mentioned great scientists, mortality rates due to TB began to decline (Grigg, 1958; Wilson, 1990; Davies et al., 1999).

In 1859, Hermann Brehmar from Germany, introduced sanatoria where he emphasized a regimen of rich diet and carefully supervised exercise as a cure for TB. However, this only lasted for a while as seventy five percent of patients who were admitted to sanatoria

were reported dead within five years. In the early 19th and 20th centuries surgical collapse (pnuemothorax) was used for TB-cure.

Three decades later, in 1945, para-aminosalicylic acid (discovered by Jorgen Lehman) and thoisemicarbazone (discovered by Gerhard Domagk) were the first antibiotic and anti-bacterial agent effective against MTB. However, these agents were bacteriostatic (Ryan, 1997). In 1944, streptomycin was discovered by American biochemists Selman Waksman, Albert Schatz, and Elizabeth Bugie (Schatz et al., 1944; Ryan, 1997; Daniel, 2000). Although many articles report that Dr Waksman discovered streptomycin, it was actually his Ph.D student Albert Schatz who first discovered streptomycin. Albert Schatz was working toward a Ph.D. degree, and was looking for an antibiotic agent possessing activity against Gram-negative eubacteria, while searching for a specific antimycobacterial agent active against the tubercle bacillus, at Selman Waksman's Department of Soil Microbiology at Rutgers University (Schatz, 1944). During this period he successfully isolated two strains of *Streptomyces griseus* from two separate sources. Both strains produced streptomycin which was found to be effective against both Gram-negative bacteria and to have bacteriostatic and bactericidal activity for the tubercle bacillus. Schatz reported that Waksman was not directly involved in any way with the early stages of his streptomycin research. In 1944, patients suffering from tuberculosis, at the Mayo Clinic were given the experimental drug. Within a week in some patients, several weeks in others, the tubercle bacillus disappeared from the sputum and for the first time in the history of plagues, the seemed to be a drug, to cure tuberculosis giving hope to millions of victims (Schatz, 1993).

The efficacy of this drug was tested in controlled studies in which 55 treated patients were compared to 52 control patients. Results from this study showed improvement in health in only 31 patients whilst 12 patients died (Medical Research Council, 1948).

In 1952, isoniazid, the first oral mycobacterial drug was discovered by the pharmaceutical company, Roche (trademarked as Rimifon®), followed by the rifampins discovery by Prof. Piero Sensi in 1957, in the Lepetit Pharmaceuticals research lab in Milan, Italy. These drugs were very effective with the result that sanatoria closed down and treatment was now available for patients infected with MTB. Isoniazid and rifamycins are still used effectively today as first line drugs, to treat drug-susceptible TB. Ever since the 1950s, when the first chemotherapeutics were isolated, there has been an explosion of research into chemotherapeutics coincident with an increase in multi-drug and extremely drug-resistant strains of TB.

1.3 TB Epidemiology

Almost one third of the world's population is infected with tuberculosis (1.7 billion people), with the developing world being most severely affected. The highest mortality rates due to tuberculosis have occurred in the last decade (Datta and Swaminathan, 2001, World Health Organization Report, 2009). In 2006, an alarming 9.2 million new active cases (4.1 million being sputum positive) corresponding to an estimated incidence of 139 per 100 000 population occurred throughout the world (World Health Organization Report, 2008; Ahmed et al., 2009). Three to nine million cases were found to be sputum smear positive and this accounted for only those cases that were reported to public health

authorities and the WHO (Dye et al., 1999; Corbette et al., 2003; World Health Organisation Report; 2008). Epidemiology studies revealed that ninety-five percent of all tuberculosis cases in the world occur in developing countries (Figure 1.4).

It has been reported that the incidence rate of TB is highest amongst young adults (generally immigrants) and most cases are due to recent infection or reinfection. There are very few cases amongst those aged between 0-14 years, but this could be due to the fact that it is very difficult to diagnose pediatric tuberculosis (Dye et al., 1999).

The highest incidence rate (363 per 100 000 population) was recorded for the African region, mainly due the the prevalence of human immunodeficiency virus (HIV) infection. The 22 high burden (11 Asian, 9 African, 1 South American and 1 East European) countries accounted for >84% of all active TB cases worldwide (World Health Organisation, 2008; Ahmed et al., 2009) The South African public health system is burdened by one of the worst TB epidemics in the world. This may be attributed to historical negligence, poor management systems and the social, economic and environmental conditions created by apartheid (Coovadia et al., 2009; Karim et al., 2009).

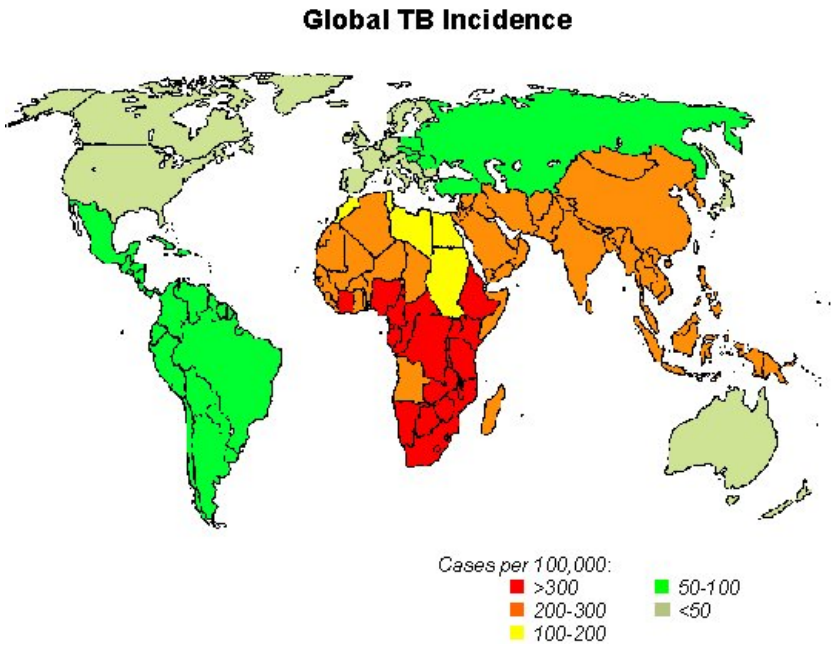


Figure 1.4 TB incidences in the world (http://ethnomed.org/clin_topics/tb/firland/epidemiology/global_tb_incidence.jpg)accessed-4/10/2008.

The World Health Organization estimated South Africa's overall annual tuberculosis rate to be 940 per 100 000 population in 2006. In 2006, South Africa reported 341 165 tuberculosis cases in the world, the fourth largest number of cases in the world, after the far more populous countries of India, China and Indonesia (World Health Organization Report, 2008). The yearly tuberculosis mortality rate increased by 2.8 times from 1990 to 2006, from 78 per 100 000 population to 218 per 100 000 population. Tuberculosis is the most commonly notified natural cause of death in South Africa (Health Systems Trust District health barometer, 2006).

In a community study in South Africa, the case notification rate in children 0-5 years old was 3588 cases/100 000, which is 3.5 times the case notification rate in adults. In this

study, the high incidence of TB in children correlated with poor parental education, low annual household income and overcrowding (Van Rie et al., 1999).

The large increase in TB cases in third world and developing countries is due to

- Economic decline
- Poor tuberculosis control
- Sub-standard health services

The spread of HIV infection in populations around the world has also had a profound effect on tuberculosis epidemiology. In 2004, 1.7 million people died of tuberculosis, including 264 000 who were co-infected with AIDS. Chronic diseases such as diabetes, undernutrition, and respiratory illnesses are other potentially important risk factors that may contribute to TB susceptibility.

Improvement of socio-economic conditions and better access to health services will help TB infection- rates to decline and possibly eradicate the causative agent MTB (World Health Organization Report, 2008).

1.4. Pathogenesis of TB

Primary tuberculosis typically appears as an “air space” disease. In primary tuberculosis, fibrosis and healing occur. However, the post-primary form of the disease tends to progress with foci of inflammation and necrosis, enlarging to occupy greater parts of the lung parenchyma (Santosh et al., 2005). The expanding infection may extend toward the

periphery of the lung and rupture into the pleural space resulting in emphysema (Kim et al., 2001; Daltro et al., 2002)

The course of the disease depends on the interaction between the host response and the virulence of the organism. The major host defense against the tubercle bacilli is cell-mediated immunity which is effected initially by macrophage activation and later by T-lymphocytes. If cell-mediated immunity is effective, there is gradual healing of the lesion with the formation of parenchyma scars (Figure 1.5) (Santosh et al., 2005).

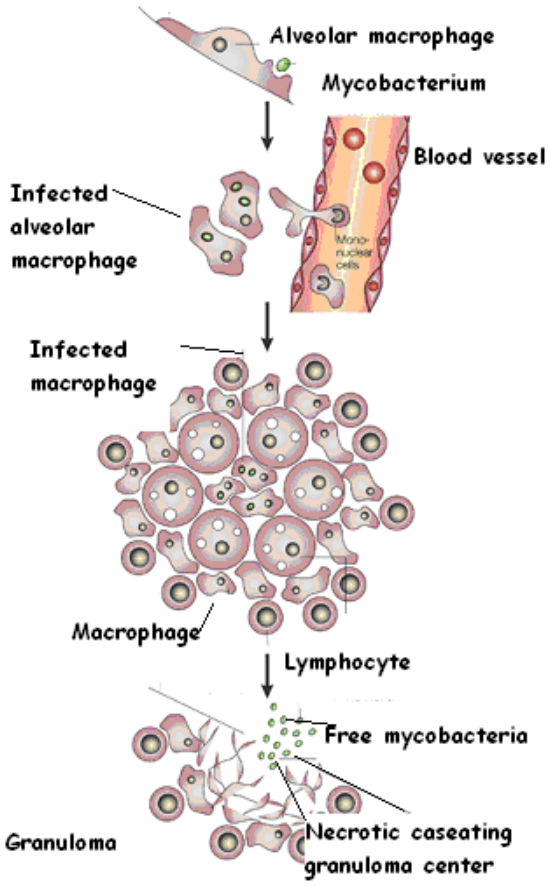


Figure 1.5 Pathogenesis of TB (www.nature.com/.../n8/images/nrm0801_569a_i1.gif)

However if MTB overpowers the host's defenses, the disease progresses to the other parts of the lung or body from the airways via lymphatic vessels or bloodstream (Santosh et al., 2005).

The inhaled bacillus may multiply or be eliminated by alveolar macrophages before any lesion is produced (Dannenberg and Tomashefski, 1998). Small lesions may stabilize if the host cellular immune defense is strong enough. However, larger lesions may be caseous and liquefy, introducing the bacilli and their products into the bronchial tree leading to extra-pulmonary dissemination of the bacilli via lymph and blood vessels.

The tubercle bacilli first lodges and begins to multiply in the lungs. The capacity of the host to control the multiplication of the bacilli, will determine whether the inhaled bacilli will be eradicated or grow into a lesion (Figure 1.6) (Carole et al., 2001).

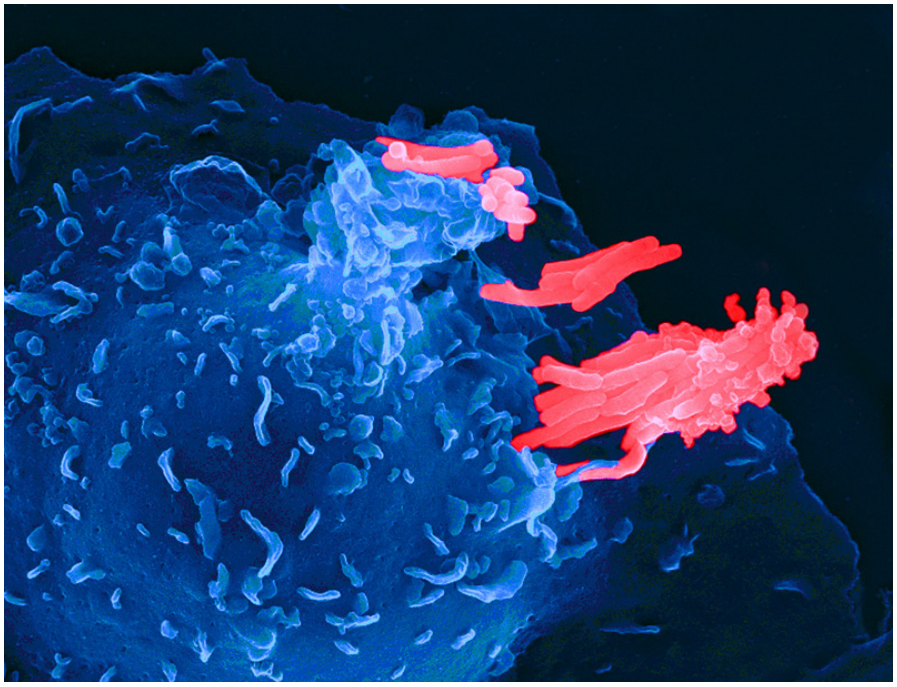


Figure 1.6 Macrophage engulfing MTB

(reference:ww.mpg.de/.../2004/10/Golinski01/Web_Zoom.jpeg)-accessed 3/06/2007

Activated macrophages are the first line of defense as they are rich in lysosomes and enzymes which aid in the destruction of the bacilli. Activated macrophages are highly phagocytic, digestive and microbicidal. Specific cell-mediated immune responses may occur after 2-4 weeks, which results in local accumulation of lymphocytes to the lungs (Dannenberg and Tomashefski, 1998). Ninety percent of all people have tubercle bacilli under control in a latent state throughout their lifetime, as a result of the immune defense system. Even after the tuberculosis infection heals, the lung may still contain bacilli that are not susceptible to sterilization by antimicrobial agents and bacilli that may persist in a dormant, metabolically inactive state. The bacilli remain viable in the host throughout life and may be reactivated with progression to active disease when cellular immune defenses

are compromised for example by old age, HIV, corticosteroids and other immuno-suppressants.

After infection, MTB may remain latent for weeks to a year before causing infection (Frothingham et al., 2005). Bacilli escape intermittently into the bloodstream and may lodge anywhere in the body, but particularly in the nervous system, bones and kidney (Carole et al., 2001).

1.5 Immune response to TB

Tuberculosis is caused by the inhalation of the airborne intracellular pathogen MTB which is contained in droplet nuclei. The respiratory bronchial epithelium (upper respiratory tract) possesses the ability to produce antimicrobial peptides (tracheal antimicrobial peptide) with a wide spectrum of activity which renders it remarkably resistant to infection by MTB (Bhatt and Salgame, 2007). These droplet nuclei of MTB which are 1 to 2 μm in size are small enough to allow passage into the lower respiratory tract. They settle in the lungs, which is the point of entry for the microorganism into the body and the primary site of the infection (Teixeira et al., 2007). Over the years, clinical evidence has shown that in addition to innate virulence of the tubercle bacillus itself, the host response to MTB plays an important role in determining the clinical manifestations and ultimate outcome of persons infected with this pathogen (Schluger and Rom, 1998; Malik et al., 2003) (Table 1.1 p18). Recent unpublished data have suggested that MTB produces biofilm. Biofilm, a self-generated polymer matrix, is an important virulence

factor that reduces antimicrobial activity of antibiotics, prevents bacterial dehydration, promotes bacterial adherence to airway epithelium and favours bacterial persistence. In the case of MTB, the biofilm appears to consist of MTB-derived mycolic acids containing nucleic acid and cholesterol released by disintegrating inflammatory cells. Cholesterol may serve as an energy source for the biofilm-encased bacilli (Prof I. Orme, MRC TB Symposium, Cape Town, June 2009).

1.5.1 Initial infection with MTB

After entry into the lungs MTB has 4 potential fates:

a) The initial host response can be completely effective and kill all bacilli, such that the patient has no chance of developing tuberculosis at any time in the future.

b) The organisms can begin to multiply and grow immediately after infection, causing clinical disease known as primary tuberculosis.

c) Bacilli may become dormant and never cause disease at all, such that the patient has what is referred to as latent infection, manifest only by a positive tuberculin skin test.

d) The latent organisms can eventually begin to grow, with resultant clinical disease, known as reactivation tuberculosis (Schluger and Rom, 1998).

1.5.2 Binding of MTB to monocytes and macrophages

Macrophages in the lungs are the first line of defense against MTB. Macrophages are capable of inhibiting the growth of the bacillus through phagocytosis, recruitment of T-

lymphocytes, and by participating in cellular immunity through the process of antigen presentation. An overview of the interactions between macrophages and mycobacteria is given in the Table 1.1 (page 47). Phagocytosis usually begins with the phagocytic cell engulfing the invading microbe and encapsulating it in a membrane- bound tight vacuole, which is created when pseudopods surround the bacterium and fuse distally (Hirota et al., 2008). The creation of this vacuole or phagosome results from the binding of MTB to the phagocyte through the complement receptors CR1, CR3, and CR4, mannose receptors and other receptors (Aderem and Underhill, 1999; Des Jardin et al., 2002). The interaction between mannose receptors on phagocytic cells and mycobacteria appears to be mediated through the mycobacterial surface glycolipid lipoarabinomannan (LAM) which is present on the cell wall of MTB (Silvana et al., 2008).

In addition to complement and mannose receptors, accumulating evidence exists for an important role for surfactant protein receptors in mediating bacterial binding. In a study reported by Zimmerli et al., (1996), it was shown that addition of surfactant protein-A (SP-A), obtained from patients with alveolar proteinosis, caused enhanced adherence of MTB; this binding was not diminished after washing away SP-A. Scavenger receptors located on the macrophage surface may also play a role in mediating binding of mycobacteria to phagocytic cells (Zimmerli et al., 1996; Cooper, 2009). When complement receptors C1, C3 and C4, as well as mannose receptors are blocked, using monoclonal antibodies, overall phagocytosis by human monocyte-derived macrophages is reduced by only 50 to 60%.

1.5.3 Fate of MTB after phagocytosis

After MTB is engulfed into phagosomes, it is subjected to killing via a variety of mechanisms, through a host of complicated interactions, mediated by cytokines, released by lymphocytes and phagocytes. Amongst the best characterized antimicrobial effector functions of macrophages are phagosome-lysosome fusion (lysosomal enzymes delivered to the phagosome), generation of reactive oxygen intermediates by the oxidative burst, the generation of reactive nitrogen intermediates, particularly nitric oxide, and apoptosis (Cooper, 2009; Lee et al., 2009) (Table 1.1).

Table 1.1 Macrophage: mycobacterium interactions (Imanishi et al., 2007)

MACROPHAGE-MYCOBACTERIUM INTERACTIONS IN THE HOST RESPONSE AGAINST TUBERCULOSIS
I. Surface binding of *M. tuberculosis to the macrophage:*
Complement receptors CR1, CR3, CR4
Mannose receptors
Surfactant protein receptors
CD14
Scavenger receptors
II. Phagosome-lysosome fusion **III. Mycobacterial growth inhibition and/or killing:**
Production of reactive nitrogen species
Production of reactive oxygen species
Apoptosis
IV. Recruitment of accessory immune cells and development of a local inflammatory response
Elaboration of cytokines, e.g., TNF, INF-γ
Elaboration of chemokines, e.g., IL-8
Toll like receptors
Antigen presentation

1.5.4 Phagososme-lysosome fusion

Phagosome lysosome fusion has been extensively studied in MTB (Bach, 2008). Mycobacteria, however, are capable of producing ammonia, which inhibits phagosome-lysosome fusion and by alkalinizing the intralysosomal contents, it diminishes the potency of the fusion complex (Schluger and Rom, 1997).

Studies by Goren et al., (1976) showed that sulfatides (derivatives of trehalose 2-sulfate, a glycolipid produced by TB) also inhibit phagosome lysosome fusion (Goren et al., 1974). The arrest of mycobacterial phagosome maturation has been shown to occur at rab-7, a small ras-like GTP-binding protein specific for late endosomes that does not accumulate on the vesicle membrane (Via et al., 1997; Sun et al., 2007; Seto et al., 2009)

1.5.5 Production of reactive oxygen species and reactive nitrogen species

The production of reactive oxygen species (superoxide anion and hydrogen peroxide) and reactive nitrogen species, particularly nitric oxide, are important in human anti-mycobacterial host defense during early infection. MacMicking and colleagues showed that in genetically altered mice that lack the ability to produce inducible nitric oxide synthase ($iNOS^{-/-}$ knockout mice), MTB replicates much faster than in wild type animals

(MacMicking et al., 2003). An interesting aspect of host defense involves the production of reactive nitrogen species and the requirement for Nramp (Natural resistance associated macrophage protein) (Blackwell, 1996). Nramp is crucial to transporting nitrite from intracellular compartments such as the cytosol to more acidic environments such as the phagolysosome where it can be converted to NO. Defects in Nramp production or function may lead to defective production of NO and increased susceptibility to MTB.

MTB has evolved strategies to avoid oxidative killing mechanisms. MTB produces catalase and superoxide dismutase, two gene products capable of degrading reactive oxygen species (Cole et al., 1998). Deficiency in the *katG* gene encoding mycobacterial catalase results in increased susceptibility to peroxidative killing (Zhang et al., 1992). The ability of mycobacteria to evade killing by either reactive oxygen or nitrogen species may be a crucial step in the establishment of latent infection.

1.5.6 Recruitment of accessory immune cells and development of a local inflammatory reponse

Other immune cells assist macrophages substantially to control the growth of mycobacteria. The interaction with other effector cells occurs in a milieu of both cytokines and chemokines. After phagosome-lysosome fusion, antigens from MTB are processed and subsequently presented to T-helper lymphocytes (Th $CD4^+$), through major histocompatibilty class II (MHC II) molecules expressed by antigen-presenting cells. $CD4^+$ helper T-cells can be separated into two phenotypic classes, Th1 and Th2 (Smith-Garvin et al., 2009). These cells are derived from Th0 or null cells.

Phenotypically Th1 cells are characterized by their ability to produce the cytokines, interferon-γ (IFN-γ), a cytokine that is capable of activating macrophages and monocytes, as well as interleukin-2 (IL-2). $CD4^+$ cells with the Th2 phenotype secrete interleukin-4, interleukin-5 and interleukin-13, cytokines involved in the recruitment of eosinophils and production of IgE (Schluger and Rom, 1998), while IL-10 is a potent anti-inflammatory cytokine. T-cell-mediated immunity amplifies the macrophage ability to kill and digest bacteria (Mogues et al., 2001; Smith-Garvin et al., 2009). Antigen-specific T cells produce several cytokines that attract and activate macrophages and cytotoxic T-lymphocytes. IFN-γ activates the antimicrobial properties of macrophages, while tumor necrosis factor, a key cytokine involved in granuloma formation, and interleukins-2, -6, -8 and interleukin-12 are involved in the recruitment and activation of inflammatory cells, including Th cells, monocytes, natural killer (NK) cells and neutrophils.

One of the most important mechanisms in the early control of MTB infection involves interactions between the cells of the innate [macrophages and NK cells] and adaptive (effector/memory $CD4^+$ T-lymphocytes) immune systems (Korbel et al., 2008). Interaction of pattern recognition receptors on alveolar macrophages with pathogen-associated molecular patterns on MTB, specifically the interaction of Toll-like receptor-2 (TLR-2) with lipoarabinomannan on MTB, results in the release of chemokines, as well as interleukins-12 and -18 (IL-12, IL-18). The chemokines attract NK cells and efffector/memory $CD4^+$ T cells, while IL-12 and IL-18 induce the release of interferon-γ (IFN-γ) by NK cells and T cells (Korbel et al., 2008). IFN- γ, in turn, increases the antimicrobial activity of MTB-infected macrophages, enabling them to eradicate this

intracellular microbial pathogen. Amplification of this mechanism may occur via the direct interaction of lipoarabinomannan with TLR-2 on the $CD4^+$ T cells, independently of the T cell receptor for antigen (Imanishi et al., 2007).

IFN-γ is one of the crucial components in the protective response against MTB (Salgame, 2005). The bactericidal capacity of the macrophage against MTB needs to be upregulated and IFN-γ is the principal and most potent mediator of this process (Salgame, 2005). Increased production of IFN-γ increases the expression of various genes in the macrophage, resulting in augmentation of antigen presentation, increased expression of immunoglobulin receptors, and recruitment of T lymphocytes that participate in the destruction of the bacteria, while promoting the production of nitric oxide and hydrogen peroxide. In synergy with tumor necrosis factor (TNF), IFN-γ activates infected macrophages, initiating an important effector mechanism of cell-mediated immunity. Studies have shown that defects in IFN-γ genes or IFN-γ receptors predispose individuals to serious mycobacterial infections (Jouanguy et al., 1996; Pai et al., 2003).

As mentioned above, other cells also involved in the immune response to MTB are cytotoxic $CD8^+$ T cells and natural killer cells. These cells display cytotoxic activity which permits them to control mycobacterial growth through the destruction of infected cells. The immune system also produces chemokines such as macrophage inflamatory protein 1-alpha (MIP-1α), MIP-2, macrophage chemoattractant proteins-1 (MCP-1), MCP-3 and MCP-5, and IFN-γ inducible protein (IP)-10, which intensify the immune

response by recruiting leukocytes (Cho et al., 2002). Extracellular bacteria are subsequently ingested and destroyed by surrounding macrophages activated by T cells.

Lastly, another potential mechanism in macrophage defense against MTB is apoptosis (Lee et al., 2009; Persson and Vainikka, 2009). Klinger and colleagues have showed that apoptosis associated with tuberculosis is mediated through down-regulation of Bcl_2 an inhibitor of cell death (Youle et al., 2008; Lee et al., 2009). T cells have been reported to lyse target macrophages by the granule-mediated, perforin-dependent mechanism of apoptosis. The mycobactericidal protein delivered by T cells has been identified as granulysin, which has been known to have a potent lethal action against a range of microorganisms (Krensky and Clayberger, 2009).

The intricate, complex interactions of the various components of the host cellular immune system need to be more vigorously investigated to characterize mechanisms and identify key immunogens which inhibit the growth of MTB. This, in turn, would accelerate the production of an effective vaccine that would eradicate TB. Clearly, macrophages play a multiplicity of roles in tuberculosis, including antigen processing and presentation, as well as effector cell functions (Silvana et al., 2001). Better understanding of initial macrophage defenses may result in important insights into the development of latent infection as evasion of these defenses may cause active disease later (Schluger and Rom, 1997).

1.6 Multidrug resistant TB

Multidrug-resistant tuberculosis is defined as *in vitro* resistance of MTB to at least two TB drugs; rifampin and isoniazid (Skenders et al., 2005; Ahmed 2009). The development of drug resistance and multidrug-resistant TB is caused by chromosomal mutations in the different genes of the bacteria. Multidrug resistant TB develops when a sequence of these mutations enables the bacteria to become resistant to 1 drug or a group of drugs eg, rifampins at a time (Petrini and Hoffner, 1999).

MDR-TB is largely a consequence of negligent human behavior (Espinol et al., 1995). Furthermore, 3 important factors e.g. poor patient co-operation, poor quality of medicine, as well as logistical problems have contributed to MDR-TB (Petrini and Hoffner, 1999). In countries with poor socioeconomic environments and weak national TB programs, deficiencies in drug control, absence of adequate drug supply and absence of compliance to the drug regimen (6 month course usually too long as patients start feeling better after a few weeks and often quit taking the drugs) and inadequate knowledge of the individual patient's strain to prescribe an appropriate combination of drugs, have further complicated the demanding task of eradicating the TB bacilli.

There is a definite need to shorten the duration of chemotherapy from 6-8 months to two-months or less to achieve better compliance. There is also a need to implement additional strategies such as early detection of new cases to reduce transmission, improved infection control in overcrowded settings, as well as proper identification of the drug susceptibility profile of the individual's exact strain of MTB. All of these strategies will aid in

combating the present situation of escalating drug resistance (Petrini and Hoffner, 1999; Skenders et al., 2005). Clearly, there is also a constant need to identify novel agents with activity against MTB equivalent to or better than that of existing agents.

1.7 XDR-TB

Despite extensive ongoing research efforts to identify and develop novel chemotherapeutics to eradicate both drug-susceptible and MDR-strains of TB, TB remains a burden on the public health systems worldwide. In the early 1990s the world was alarmed when it was faced with yet another health crisis- the emergence of a strain of TB called extensively drug resistant tuberculosis (XDR-TB).

Extensively drug resistant tuberculosis renders patients untreatable with current anti-TB chemotherapeutic agents. It currently costs the South African government R376.74 per patient to treat drug-responsive TB (over a period of six months), R30 717.64 for MDR TB for six months, and R82 239 for XDR-TB (over a 24 month period) ($1= R8). Ethionamide and cycloserine are the only drugs available in South Africa to treat XDR-TB and was being administered at King George V Hospital in Durban where the XDR-TB crisis was most severe in 2007. Other drugs recommended for the treatment of TB are not yet available in the country with the exception of those being evaluated in clinical trials such as moxifloxacin and gatofloxacin (Thaver, 2006).

In the period 2000-2004, the Center for Disease Control and WHO surveyed TB laboratories on an international basis to assess the number of cases of MDR and XDR-TB. Two percent of the 17 690 TB patient isolates were XDR-TB. Extensively drug-resistant tuberculosis does not respond to first and second line drugs and there is no single drug available for immediate use to combat this worsening resistance. In 2000, the Center for Disease Control in the United States reported that 64% of patients affected by XDR-TB died (Center for Disease Control, 2006). The drugs currently not available in South Africa for the treatment of TB is capreomycin, prothionamide and *p*-aminosalicylic acid (http://www.doh.gov.za/tb/docs/mdrtb.html).

During the period 2003 to 2005 a study conducted at the National Research Institute of TB and Lung Diseases in Tehran, Iran showed that 10.9% of MDR strains tested were resistant to all 8 second line drugs tested. These strains were identified as belonging to the MTB superfamily Haarlem 1 and East African Indian (Masjedi et al., 2006).

Recently, and as mentioned above, the Tugela Ferry region in Kwa-Zulu Natal, South Africa was faced with a crisis, when 53 patients were diagnosed with XDR-TB. An alarming 52 of these 53 patients co-infected with AIDS died. These devastating mortality rates caused by XDR-TB, coupled to the increase in HIV/AIDS, pose a great threat to mankind (Koenig, 2008).

Other countries affected by XDR-TB include the United States (1993-2004), Latvia (2000-2002) and South Korea (2004) were 4 %, 19 % and 15% of MDR TB cases,

respectively were XDR-TB (Center for Disease Control and Prevention) (Figure 1.7). A World Health Organization report from 46 countries has revealed that 5.4 % of MDR-TB cases were found to have XDR-TB. Eight countries reported XDR-TB in more than 10% of MDR-TB cases, with the highest prevalence being in countries located in Eastern Europe and Central Asia.

(http://whqlibdoc.who.int/publications/2010/9789241599191_eng.pdf)

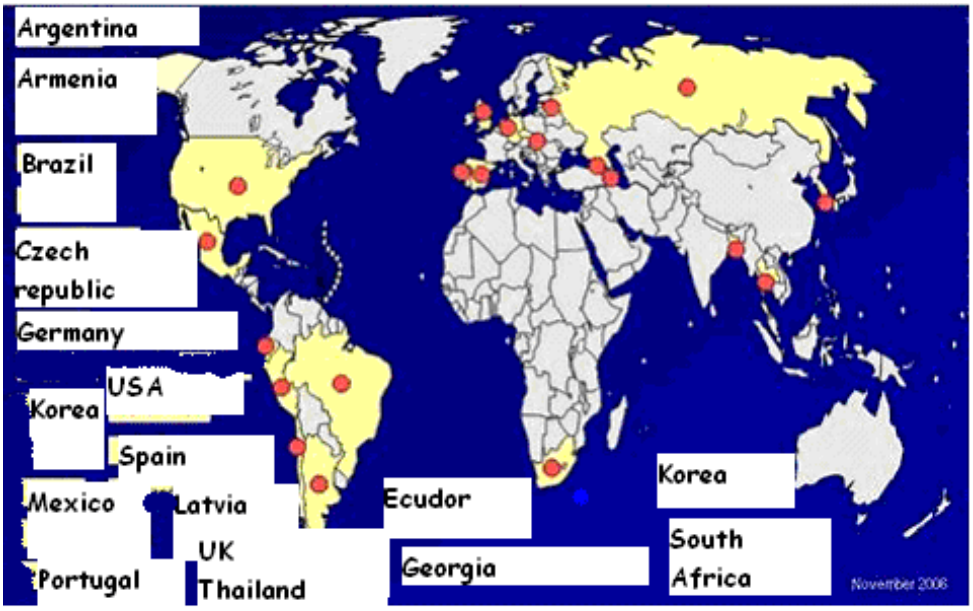


Figure 1.7 Countries with XDR-TB-confirmed cases to date (http://www.hhs.gov/asl/testify/2007/03/070321fig1.JPG)

In an effort to curb the spread of XDR-TB, the South African Medical Research Council, WHO and the US Centers for Disease Control and Prevention in TB have documented the following seven point plan:

- Rapid surveys to assess the current prevalence of XDR-TB globally
- Enhanced local laboratory capacity to carry out culture and drug resistance testing
- Increased training of public health staff to identify, investigate and treat XDR-TB outbreaks
- Implementation of infection control precautions
- Increased research support for drugs to treat XDR-TB
- Development of rapid diagnostic tests for TB
- Access to antiretroviral drugs (Center for Disease Control, 2006)

The emergence of XDR-TB poses the threat of developing into an epidemic that plagues mankind, underscoring and highlighting the need for a more aggressive focus on new anti-TB regimens, better diagnostic tests, and stricter policies with regard to control and detection strategies.

1.8 Treatment of tuberculosis

Contained within a tuberculosis lesion, there are several populations of MTB that differ with respect to metabolic activity and susceptibility to anti-tuberculosis agents (Mitchison, 2006). Large numbers of actively metabolizing MTB are found on the alkaline surface of pulmonary capillaries, where there is ample oxygen, while deeper

within the walls in caseous tissue and within macrophages there are fewer numbers of bacilli which are either intermediately metabolically active, or dormant. Actively metabolizing bacteria are relatively easy to kill, but the major problem lies with intermittently active or dormant bacilli, and failure to eliminate these leads to relapse (Donald and Schaaf, 2007).

The five main objectives of chemotherapy are to:

- Cure the patient
- Prevent death from active disease or its late effects
- Avoid relapse
- Prevent spread of drug-resistant organisms
- Protect the community from infection (Harries , 1997)

The treatment regimen for pulmonary TB has been divided into the intensive phase and maintenance phase.

The objectives of the intensive phase are to:

- Make the patient non-infectious by rapidly killing MTB
- Forestall dissemination of the disease
- Prevent drug resistance

The maintenance phase is directed towards clearing the infection and to achieve complete healing of the lesion.

Over the years it has been noted that MTB is a tough and resilient organism that is well-adapted to prolonged residence in its human host. Many therapeutic agents such as gold, calcium, iodine, quinine, turpentine, cod liver oil and derivatives of arsenic, mercury and creosote, have failed to arrest or reverse the progression of TB. Protected by a waxen cell wall, the TB bacilli are shielded against lethal enzymes and substances released from the immune system of the host in order to arrest the disease (Murray, 2004).

The first chemical identified with the ability to cross the cell wall of the TB bacilli was 'Promin' and its derivative Promizole. Trials in humans began in 1941 but abruptly came to a halt due to significant side-effects (Feldman et al., 1941). A soil microbiologist, Selman Waksman discovered the first antibiotic streptomycin (Comroe and Pay, 1978). In 1944, streptomycin was tested against 22 different species of bacteria including MTB (Schatz et al., 1944).

However, a scientist named Ryan (Ryan, 1992) observed that streptomycin was tested only on harmless strains and actually should have been tested on the virulent H37Rv strain. Fifteen months later, streptomycin was tested on a young woman dying from progressive pulmonary tuberculosis. She survived, and lived a fulfilling life thereafter (Daniel, 1997). However, streptomycin had significant 8th nerve toxicity and its benefits became short-lived as the bacteria became resistant (Hinshaw et al., 1946).

Fortunately, at around the same time, paraminosalicyclic acid (PAS) was discovered by Jorgen Lehman in Sweden and was tested on humans in October 1944. The first patient to receive PAS made a dramatic recovery. Like streptomycin, MTB developed resistance to

the drug after a short while (Medical Research Council, 1949). However, when administered together, streptomycin preserved the potency of PAS by preventing tubercle bacilli from becoming resistant to it and vice versa.

In 1951, the most extraordinary pharmaceutical co-incidence of all time occurred, when it turned out that scientists at the three pharmaceutical giants, Bayer Chemical in Germany and at Squibb and Hoffman La Roche in the United States, discovered isoniazid, a drug that was potent, safe and inexpensive. When administered to patients as a cocktail of isoniazid, streptomycin and PAS, patients suffering with TB could be cured. Streptomycin was administered only for a few months, and the other two antibiotics for a total of 18 to 24 months (Crofton, 1959).

Sanatoria closed and procedures such as pneumothorax and pneumoperitoneum became obsolete. In 1960, rifampin became available, while reappraisal and inclusion of pyrazinamide allowed the development of modern short-course antituberculosis therapy (Fox et al., 1999). After the discovery of these chemotherapeutics, TB could be treated entirely on an outpatient basis without hospitalization.

Drugs used to treat TB can be bactericidal (kill bacteria) or bacteriostatic (slows down growth). Isoniazid, rifampin, pyrazinamide and streptomycin are predominantly bactericidal, and ethambutol and thiacetazone are bacteriostatic (Mitchison, 1984).

The bacterial population in a TB patient consists of:

- Metabolically active, continuously growing bacteria found within the walls of the tuberculosis cavities
- Bacteria inside cells and macrophages
- Semi-dormant bacteria
- Dormant bacteria which die off gradually (World Health Organisation, 2007)

We therefore need drugs that target different aspects of the bacterial population to help prevent infection from spreading. The section on chemotherapy of tuberculosis (later in the chapter) deals with different drugs, known as first-line and second-line antibiotics that are either bactericidal or bacteriostatic for MTB (World Health Organisation, 2007).

This year, the World Health Organization presented a document outlining a control strategy and policy to combat this disease (World Health Organization, 2009-whqlibdoc.who.int/publications/2009/9789241598323). There are five essential components outlined in this document which aim to reduce mortality, morbidity and transmission of the disease.

The TB control policy package includes:

- Government commitment to a TB program aiming at nationwide coverage
- Case detection through passive case finding
- Short-course chemotherapy for all smear-positive TB cases
- Regular supply of essential anti-TB drugs and diagnostic material
- A monitoring and evaluation system

The success of the TB control strategy will depend on effective implementation of the TB control policy package mentioned above

The targets for TB control are

- To cure 85% of the detected new cases of smear-positive TB
- To detect 70 % of the existing smear-positive TB cases

Effective TB control requires a combination of medical expertise and commitment to the TB control policy package by the different role-players. Global TB control that will reduce mortality rates will need basic tools such as aggressive-case finding strategies, reliable and consistently funded public health treatment systems, and adherence to multi-drug treatment plans (Harries, 1997).

1.9 Prevention of TB

Prevention of tuberculosis will play an important role in combating this infectious disease. There are 3 important control strategies that deal with prevention of TB. These include:

a) Bacillus Calmette Guerin (BCG) vaccination

b) Isoniazid preventative therapy

c) Prevention of TB in hospital patients and health care workers (Harries, 1990)

Infection with MTB depends on the efficiency of the host cell immune response and the virulence of the organism. The BCG vaccine is a live vaccine containing several strains of *M.bovis*. It was administered for the first time in 1921 and is currently used by a number of countries as part of childhood routine immunization (Harries, 1990). Currently, BCG vaccine is administered to 100 million children each year throughout the world. The efficacy of BCG is based on worldwide clinical trials which have shown that BCG vaccine protects against TB-meninigitis and miliary TB in immunocompetent children (ten Dam, 1993; Colditz et al., 1994; Comstock, 1994; Rodrigues et al., 1995). However, it does not prevent primary infection with MTB.

Another preventative measure is the use of chemoprophylaxis to prevent tuberculosis. Isoniazid is given prior to exposure or even after exposure to prevent development of overt disease. Isoniazid is believed to be effective in such situations by diminishing or destroying small bacterial populations in infected patients, thereby preventing the development of active disease (Harries, 1990). Rifampin and pyrazinamide have bactericidal and sterilizing properties and have been used in conjunction with isoniazid to prevent TB in breast-fed mothers of sputum-positive individuals, newly-infected patients, and clinical states in which TB is most likely to develop i.e. HIV infection, Hodgkin's disease and diabetes mellitus. Another preventative measure to combat the spread of TB is protection of health care workers and patients in healthcare settings i.e. nosocomial (Edlin et al., 1992; Sepkowitz, 1995; Wenger et al., 1995)

To prevent unnecessary spread of the disease the following guidelines have been issued by the WHO in 2009:

- Isolation of infectious tuberculosis patients from other patients while diagnosis is still being established- a TB ward must be established and patients with smear-positive sputa should be isolated from patients who are immunocompromised.
- Environmental control-proper ventilation is one of the effective ways of preventing spread of tuberculosis using wide windows and ample ultraviolet light.
- Protecting health care workers who are immunocompromised-health care workers who are HIV-infected should not be exposed to infectious TB patients and should not work with TB patients. Also, masks should be used to prevent aerolization from patients who cough and sneeze (World Health Organization Report, 2009).

1.10. The impact of TB on the HIV/AIDS epidemic

HIV has become the most perilous risk factor that enhances the progression of tuberculosis infection to clinically active TB. This is irrespective of whether the HIV-infected individual was recently infected or the infection was latent (Murray, 1998). TB has been described as the single most important opportunistic HIV-related disease and is the leading worldwide cause of morbidity and mortality among people with HIV/AIDS (Murray, 1998). At the beginning of the century, 8.3 million new cases of TB emerged with 9% attributable to co-existing HIV infection and the cases were a substantial 31% higher in sub-Saharan Africa (World Health Organization Report, 2001). Poverty, poor political commitment, ineffective follow-up of 'directly observed treatment strategy'

treatment, poor diagnosis and unreliable supply of antibiotics has worsened the situation in HIV-infected individuals co-infected with TB. HIV-infection has become the main driving force in the global resurgence of TB (Murray, 2004).

In the Hlabisa district of South Africa, as the HIV prevalence rate increased, so did the number of TB deaths and case fatality rate (Andrews et al., 2007). The TB fatality rate increased from 9.2% in 1991 to 13.4 % in 1995; the average number of TB deaths per month more than doubled between 1991/1992 and 1995. TB patients with known HIV infection were more likely to die than TB patients who were HIV-negative (Corbett et al., 2003). In 2006, approximately 246 000 HIV-infected individuals with TB died.

1.11 Mechanisms of action of existing Anti-tuberculosis drugs:

Currently, the five essential drugs recommended by WHO are isoniazid, rifampin, pyrazinamide, streptomycin and ethambutol (Donald and Schaaf, 2007; http://www.merck.com/mmpe/sec14/ch179/ch179b.htm). These five drugs are known as first-line antituberculosis agents and have been used in treatment regimens for over thirty years. Aminoglycosides: e.g., amikacin, kanamycin; polypeptides, e.g., capreomycin, viomycin, enviomycin; fluoroquinolones: e.g., ciprofloxacin, levofloxacin, moxifloxacin; thioamides: e.g. ethionamide, prothionamide, cycloserine and p-aminosalicylic acid are second- line agents (Center for Disease Control, 2006). Targets of current drugs include cell wall synthesis (isoniazid, ethionamide, ethambutol and cycloserine), folate synthesis (p-aminosalicylate), transcription (rifampin), translation (aminoglycosides), DNA

(deoxyribonucleic acid) metabolism (fluoroquinolones) and the cell membrane (pyrazinamide). Three new compounds affect other bacterial targets i.e. TMC207, OPC-67863 and PA-824. TMC207 seems to inhibit the ATP synthase complex. OPC-67863, and PA-824 are prodrugs, the activation of which depends on the same cellular enzyme Rv3547 (see Figure 1.8).

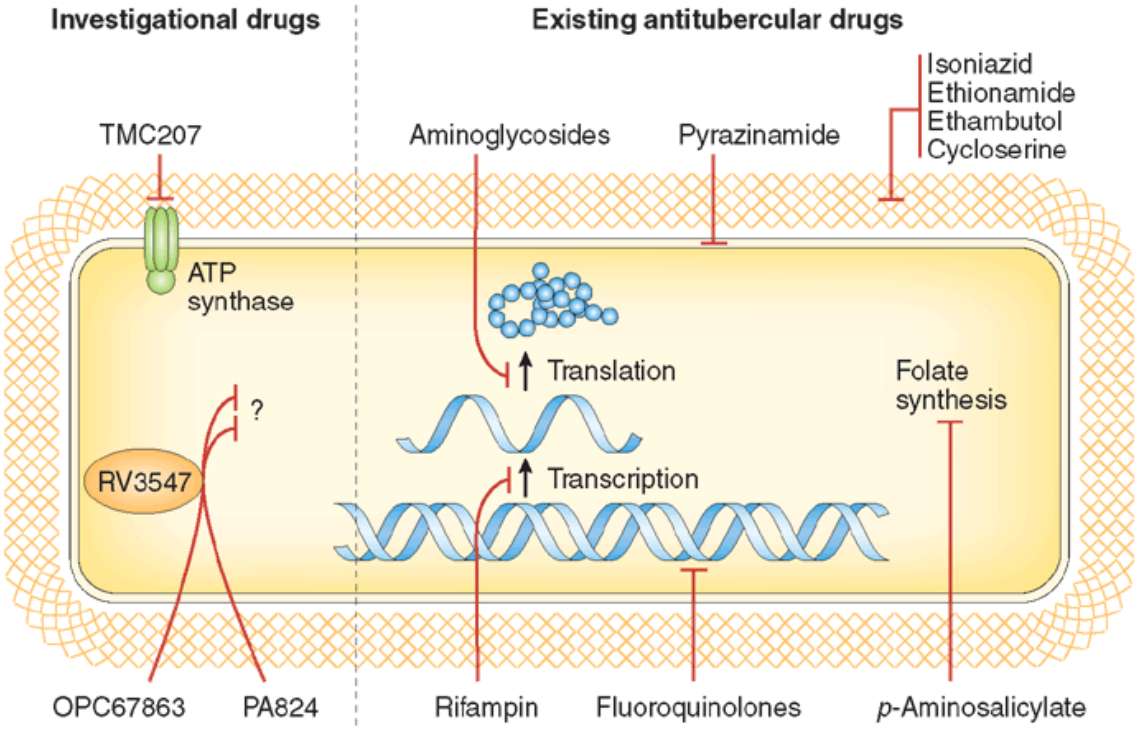


Figure 1.8 Mechanisms of action of current and investigational tuberculosis drugs. (www.nature.com/.../n3/images/nm0307-279-F1.gif)

These drugs work differently on mycobacteria i.e. they may be bactericidal or act as a sterilant (Harries, 1997). Bactericidal activity refers to the agent's ability to rapidly kill the actively metabolizing organisms present in the airways of adult patients with

pulmonary tuberculosis. Sterilizing activity describes an agent's ability to prevent relapse by killing all remaining viable bacilli in the patient's lesions (Donald and Schaaf, 2007).

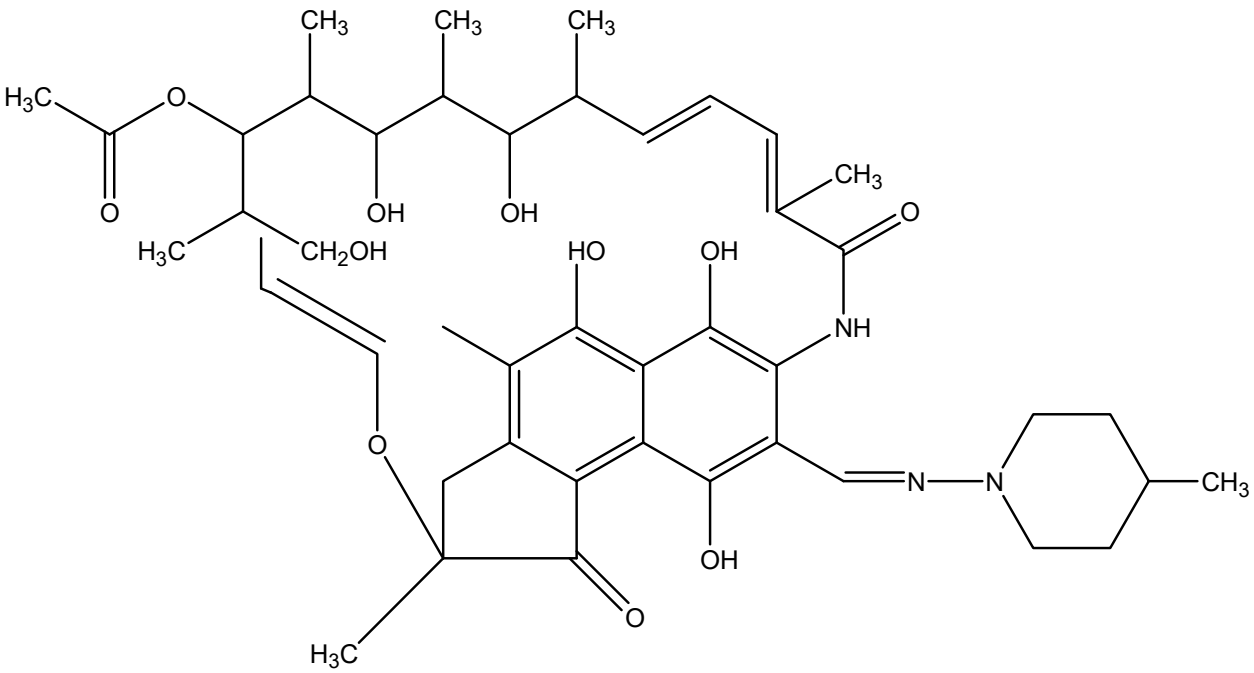
1.12 Description of first- and second-line TB-drugs

1.12.1 Rifampin

Rifampin (RMP) has good bactericidal properties. It has a sterilizing action and is particularly active against semi-dormant bacilli, i.e. bacilli which undergo spurts of metabolism when the local environment changes. These bacteria escape the bactericidal action of INH and may cause relapse after treatment. Rifampin prevents the growth of these bacteria during relapse (Harries, 1997).

Rifampin is a prototype of a class of antibiotics of the rifampin class. Rifampin has been an integral part of well-established combination regimens for the treatment of MTB. Rifampin is currently marketed as Rifadin, Rifar and Rimcutane by the pharmaceutical company, 'Aventis'. Rifampin inhibits DNA-dependent RNA polymerase in bacterial cells by binding to its beta subunit and thereby preventing transcription of messenger RNA and subsequent translation to proteins, with little activity against the equivalent mammalian enzymes (Levin, 1993).

Rifampin is a water soluble reddish-brown crystalline powder. Rifampin is inhibitory against MTB at levels as low as 0.005ug/ml. It kills bacteria that are metabolically dormant. Rifampin is also active against *M. bovis, M. kanssaii, M. marinum* and *M. leprae.*



Rifampicin

Figure 1.9 Chemical structure of rifampin (www.drugs.com/pro/images/0c7adf7f-d2c3-4b3a-b)

1.12.2 Pyrazinamide

Pyrazinamide (PZA) was first introduced in 1952 and produced impressive sterilization in both animal and clinical experiments (McCune et al., 1956). Pyrazinamide is an

important sterilizing agent that has the unique ability to kill bacilli that are well protected in an acid medium inside the macrophages.

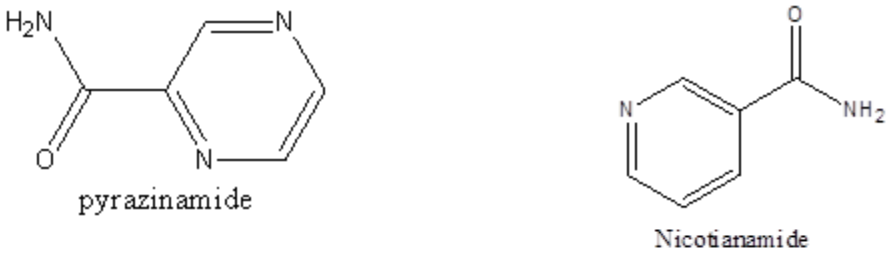


Figure 1.10 Chemical structure of pyrazinamide and nicotinamide

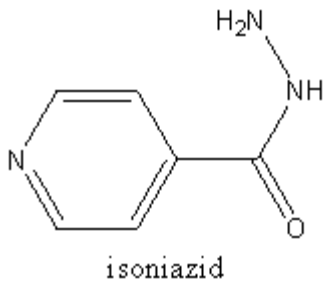
Its activity decreases as the growth rate of the organism declines so it becomes ineffective in organisms that have slow growth rates (Mitchison, 1979). Pyrazinamide is administered in the first 2 months of treatment (initial phase of treatment) and any further administration of this drug does not improve the relapse rates after chemotherapy (Mitchison, 1979; Harries, 1997).

Pyrazinamide is a synthetic pyrazine analog of nicotinamide. PZA is a prodrug that requires activation or conversion to its active form, pyrazinoic acid (POA) by the PZase/nicotinamidase enzyme which, is found within MTB and hydrolyzes pyrazinamide to pyrazinoic acid. The target of POA appears to be the bacterial cell membrane (Zhang et al., 2002).

The minimal inhibitory concentration of PZA is strongly influenced by pH (Salfinger, 1988). It is very specific and has an MIC of 50µg/ml (Lutwick, 1995). PZA has good pharmocokinetics and is able to penetrate most tissues including cerebrospinal fluid (Ellard et al., 1993). Recent testing on highly resistant strains of MTB revealed that PZA resistance is uncommon (World Health Organization Report, 2003).

1.12.3 Isoniazid

Isoniazid, also known as isonicotinic acid hydrazine (INH), is a potent bactericidal drug discovered in 1945. It is an inexpensive, well-tolerated drug and a very valuable agent for the treatment of tuberculosis. INH has been the cornerstone of antituberculosis regimens because it has a high early bactericidal activity and relatively low toxicity.



isoniazid

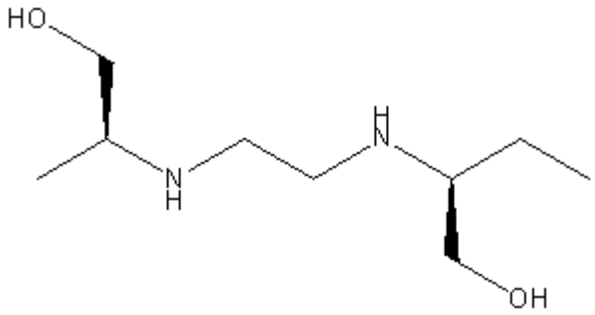
Figure 1.11 Chemical structure of isoniazid

INH rapidly eliminates metabolically active bacilli present in tuberculosis cavities and when used in combination with RMP and PZA it protects these drugs from resistance (Donald and Schaaf, 2007).

INH is a prodrug and requires activation from the inactive form by the mycobacterial enzyme catalase-peroxidase. INH prevents mycolic acid biosynthesis by inhibiting a 2-trans-enoyl-acyl carrier protein reductase that belongs to the fatty acid synthase II system. (Marrakchi et al, 2000). Mycolic acid makes up an integral part of the cell wall of *M. tuberculosis* (Yepes et al., 2004). Peroxidation of endogenous lipids has been shown to be a major factor in the cytotoxic action of INH (Santhosh et al., 2007). The minimal inhibitory concentration (MIC) of INH for the tubercle bacillus is 0.025-0.5ug/ml (Lutwick, 1995).

INH is currently the most potent bactericidal drug and kills 90% of the bacillary population during the first few weeks of chemotherapy. It is said to be most effective against continuously growing bacteria. INH acts exclusively against mycobacteria, including *M. bovis* and *M. africanum* (Reichman and Hersfield, 2000).

1.12.4 Ethambutol



Ethambutol

Figure 1.12 Chemical structure of ethambutol

Ethambutol (EMB) was discovered in the early 1960s and was first reported by Wilkinson and co-workers (Wilkinson et al., 1961). In tuberculosis treatment regimens it is regarded as one of the most reliable first line anti-mycobacterial chemotherapeutic agents. EMB has moderate bacteriostatic activity. It is used in combination with INH, rifampin and PZA (Lety et al., 1997; Faugerox et al., 2007).

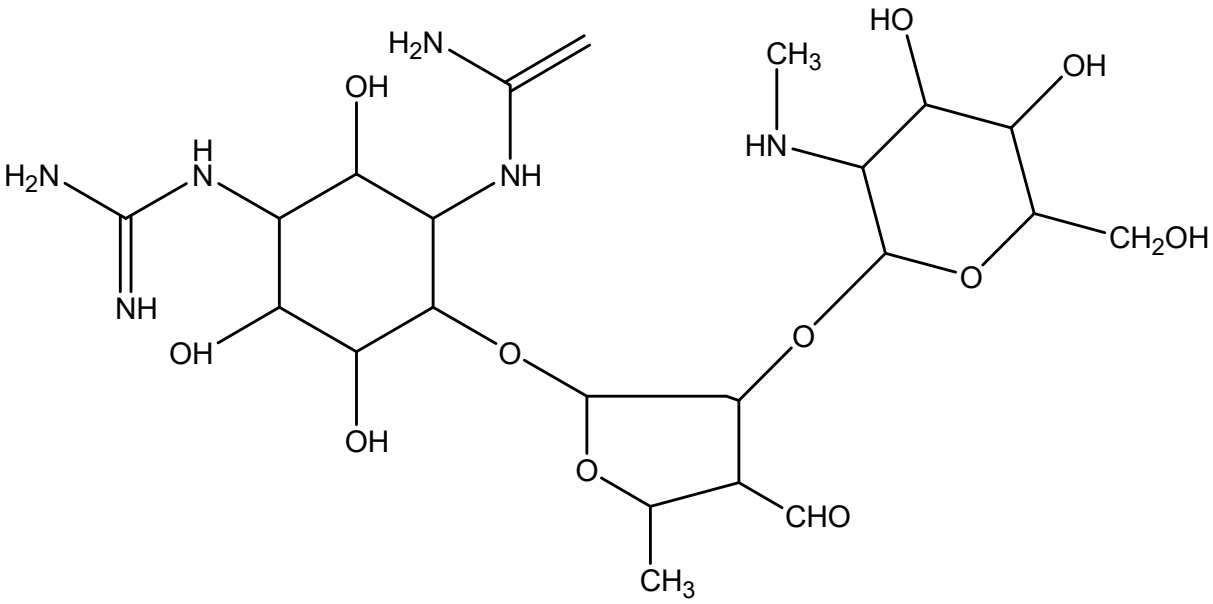
Ethambutol appears to exert its effects on MTB by inhibiting the biosynthesis of the mycobacterial cell wall (Lee et al., 1995). EMB primarily acts on arabinosyltransferases responsible for building the arabin core of both the mycolyl-arabinogalactan and liparabinomannan units, thereby inhibiting the incorporation of mycolic acid into the cell wall (Faugerox et al., 2007). It generally acts on metabolically active cells. The MIC of EMB is 2 µg/ml and it is effective against almost 90% of mycobacterial strains.

1.12.5 Aminoglycosides

Aminoglycosides are known as aminoglycosidic aminocylitols which are made up of two or more amino sugars attached by glycosidic bonds. Streptomycin and kanamycin are two very important aminoglycosides used in the treatment of TB (Figure 1.13). Streptomycin, was first discovered in 1944 by Waksman (Singh, 1954). Kanamycin, has two isomers but only kanamycin A has anti-TB activity. The biological activity of kanamycin is pH–dependent and this agent works optimally at higher pH values. Aminoglycosides are bactericidal to TB bacilli. Streptomycin binds to a single site on the bacterial ribosome.

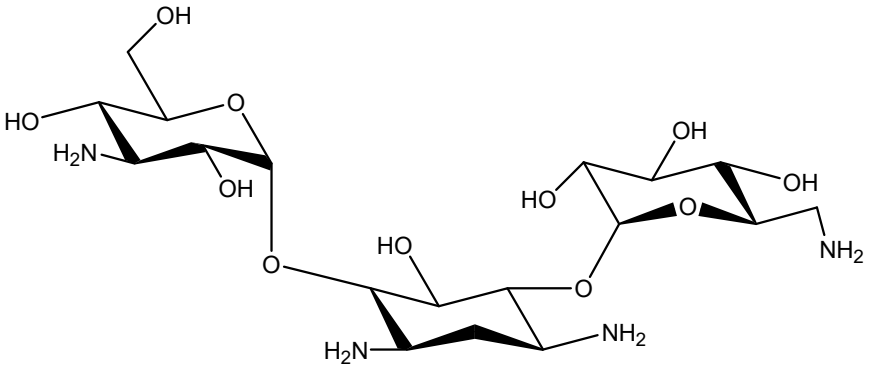
By doing this, it blocks the initiation stage in translocation and prevents polypeptide elongation. Kanamycin, however, binds to multiple sites on the ribosome. Resistance to aminoglycosides may arise through mechanisms which alter ribosomal binding sites.

a)



Streptomycin

b)



Kanamycin

Figure 1.13 Structure of second line agents used to treat tuberculosis a) Streptomycin b) Kanamycin

2.1 Introduction

Plants produce a large variety of organic compounds known as secondary metabolites. These compounds appear to have no direct function in growth and development, but are used as defense mechanism against herbivores and pathogenic microbes (Taiz and Zeiger, 2001). Plant greens and seeds were important foods in the traditional diet of European farmers (Guil et al., 1998). Today these plants are used not only as food, but also combination of these plants are prescribed by traditional healers for treatment of diseases ranging from the common cold to malaria (Obiajunwa et al., 2002).

2.2 Uses of natural products

Many people living in Afro-Asian countries rely on indigenous medical systems for the treatment of ailments. Roots, leaves, fruits and flowers of different plant materials, as well as marine plants and animals are used in concoctions to cure ailments. Years of research, including isolation, structural and synthetic studies have been directed to the discovery of new natural products. A number of products isolated from indigenous medicinal plants have proved to be an invaluable source of novel secondary metabolites which possess a wide diversity of pharmacological properties and a broad structural diversity (Obiajunwa et al., 2002).

Many traditional healers use plants in their crude form (herbal remedies) to treat ailments. Today there is a growing interest in natural and traditional medicines and scientists all over the world are looking for new cures in collaboration with traditional healers (Sindambiwe et al., 1999). The use of traditional medicine in most countries is embedded in a complex ethno- medical system that co-exists with official medicine or biomedicine, alternative medicine and religious therapies (Idoyaga, 2001). Over 80 % of the population in developing countries depends on plants for their medicinal requirements (World Health Organization Report, 2002). In these countries, traditional medicine is the key element for the provision of healthcare, especially in rural settings, where there are inadequate healthcare systems (Tabuti et al., 2003).

2.3 Traditional medicinal plant use in South Africa

In South Africa more than 60% of the population uses indigenous medicinal plants for their health care needs and cultural practices. There are approximately 3000 species of plants that are used by an estimated 200 000 indigenous traditional healers (Van Wyk and Van Wyk, 1999). Seventy percent of South Africans prefer to consult traditional healers known as ‘inyanga’ than to consult modern medical practitioners. The ‘inyanga’ uses many traditional medicines to treat various ailments, as well as psychosocial illnesses (Table 2.1). Parts of the plant are prepared as powders that are used like snuff or, in the form of smoke or fumes, or infusions (tinctures), or applied topically to wounds.

Medicinal plants being cultivated for the international market include *Warburgia salutaris* (pepperbark tree), *Siphonochilus aethiopis* (African ginger), *Aloe ferox* (Cape aloes), *Agathosma sp*. (buchu), *Harpagophytum procumbens* DC (devil's claw), *Pelargonium sidoides* DC (Umkcaloabo) and *Xysmalobium undulatum* (Uzara) (McGaw et al, 2008). South Africa boasts so-called 'green gold', meaning a large number of plant species that contain invaluable chemical components that have the potential to play a role in the medicinal market on a global scale (Figure 2.1).

Table 2.1: Traditional medicinal plants used by South Africans to treat ailments (Mativandlela, 2005)

Species	**Family**	**Popular name**
Agathosma betulina	Rutaceae	Buchu
Agasthoma crenulata	Rutaceae	Buchu
Aloe ferox miller	Asphodelaceae	Aloe
Artemisia afra Jacq. Ex Wild	Asteracea	Wormwood
Balanite maughamii Delile	Belanitaceae	Torchwood
Bersama tysoniana Oliv	Melianthaceae	White ash
Boophane disticha (Herbert)	Amaryllidaceous	Tumbleweed
Bowiea volubis Harv	Hyacinthaceae	Climbing lilly
Cassine papillosa (Hochst) Kuntze	Celastraceae	Common saffron
Clivia miniata regel	Amaryllidaceous	Bush lilly
Crytptocarya Latifolia sond	Lauraceae	Broad leave quince
Curtisia dentate9 Diosorea sylvatica Ecklon	Cornaceae	Assegai
Euconomis autmnalis (Mill) Chitt	Dioscireaceae	Elephants foot
Gunnera perpensa L	Hyacinthaceae	Wild pineapple
Harpagophylun procumbens	Gunneraceae	Wild rhubarb
Ocoteabullata (Burchell) bailon	Lauraceae	Devil's claw
Perlargonium sidoides	Generaniaceae	Stinkwood
Pittosporium viviflorum sims	Pittosporaceae	Umkealoabo

Rapanea melanophloes (l) Mez	Myrsinaceae	Cheesewood
Scilla Natalensis Planch	Hyacinthaceae	Cape beech
Siphono chilus aethiopicus(Schweinf) B.L Birtt	Zinigiberaceae	Blue hyacinth
Stangeria eriopus Nash	Stangeriaceae	African ginger
Warburgia salutaris (Bertol F Chiov	Canellaceae	Natal grass cycad

Figure 2.1 Medicinal plants trade in Russell Street, Durban, South Africa (www.fao.org)

2.4 Medicines derived from natural products

Over the years, pharmaceutical companies have developed some plant products into more palatable forms like tablets, capsules and syrups (Ndbani and Hojer, 1999). About 25% of prescribed drugs used in modern medicine originate from the plant kingdom (Ballick, 1990; Harvey, 2000) and one-third of the top selling drugs in the world are natural products and their derivatives (Table 2.2).

Plants investigated for their medicinal properties have yielded various compounds which are used at present for medicinal purposes. For example, many years ago a plant chemical was discovered from a tropical plant, *Cephaelis ipecacuanha.* The chemical entity was named ipecac and is used to induce vomiting if a person swallowed a poisonous and harmful substance.

Another example of a plant-derived drug is a very effective anticancer drug, taxol isolated from the Pacific Yew tree, *Taxus brevifolia.* A pharmaceutical company modified this and patented a drug named Paclitaxel ™ which is used worldwide today. Vinblastine and vincristine are two important chemicals derived from the *Madagscar periwinkle* which have been used as first choice drugs for the treatment of various forms of leukemias since the 1950s. Topotecan and Irinotecan are analogs of a plant alkaloid discovered from the Chinese tree, '*Campthoeca acuminate'*, which have been used for the treatment of metastatic colorectal cancer for many years. Etoposide and teniopside, semisynthetic derivatives of a plant chemical discovered from *Podophyllum peltatum*, have been used to treat cancers. Homoharringtonine from the Chinese tree,

Cephdysoxylum binectiferum alotaxus harringtonia, perillyl alcohol, and flavopiridol based on a flavone isolated from *Dysoxylum bineciferum,* are currently being assessed in clinical trails for the treatment of several types of cancers **(**http://www.rain-tree.com/plantdrugs.htm-acessed 1/9/09**).**

Table 2.2 The world's best selling pharmaceuticals in 2004 (Harvey, 2000)

Position	**Product**	**Therapeutic Class**	Sales $ m
1.	Ranitidine	H_2 antagonist	3,032
2.	Enalapril	ACE inhibitor	1,745
3.	Captopril	ACE inhibitor	1,580
4.	Diclofenac	NSAID	1.185
5.	Atenolol	ß-antagonist	1.180
6.	Nifidipene	Ca^{2+} antagonist	1.120
7.	Cimetidine	H_2 antagonist	1.097
8 .	Mevinolin	HMGCoA-R inhibitor	1,090
9.	Naproxen	NSAID	954
10.	Cefactor	ß-lactam antibiotic	935
11.	Dilitiazem	Ca^{2+} antagonist	912
12.	Fluoxetine	5HT reuptake inhibitor	910
13.	Ciprofloxacin	Quinolone antibiotic	904
14.	Amlodipine	Ca^{2+} antagonist	896
15.	Amoxycillin	ß-lactam antibiotic	892
16.	Acylcovir	Anti-herpetic	887
17.	Ceftriaxone	ß-lactam antibiotic	870
18.	Omeprazole	H^+ inhibitor	775

19.	Terfenadine	Anti-histamine	768
20.	Salbutamol	ß$_2$-antagonist	757

Quinine an active plant ingredient from the South American *Cinchona tree*, atovoquone and artemisinin from the plant *Artemisia annua* (Sweet wormwood) have been used as antimalarial agents and have had a major impact on reducing the number of deaths from malaria. Many other plant-derived therapeutic agents and their clinical applications are tabled in Table 2.3.

Table 2.3 Plant-derived drugs and their Clinical Application (http://www.rain-tree.com/plantdrugs.htm-acessed **1/4/08)**

Drug/Chemical	**Action/Clinical Use**	**Plant Source**
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis vernalis*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Aesculetin	Anti-dysentery	*Frazinus rhychophylla*
Agrimophol	Anthelmintic	*Agrimonia supatoria*
Ajmalicine	Circulatory Disorders	*Rauvolfia sepentina*
Allantoin	Vulnerary	*Several plants*
Allyl isothiocyanate	Rubefacient	*Brassica nigra*
Anabesine	Skeletal muscle relaxant	*Anabasis sphylla*
Andrographolide	Baccillary dysentery	*Andrographis paniculata*
Anisodamine	Anticholinergic	*Anisodus tanguticus*

Anisodine	Anticholinergic	*Anisodus tanguticus*
Atropine	Anticholinergic	*Atropa belladonna*
Benzyl benzoate	Scabicide	*Several plants*
Berberine	Bacillary dysentery	*Berberis vulgaris*
Bergenin	Antitussive	*Ardisia japonica*
Betulinic acid	Anticancerous	*Betula alba*
Borneol	Antipyretic, analgesic, antiinflammatory	*Several plants*
Bromelain	Anti-inflammatory, proteolytic	*Ananas comosus*
Caffeine	CNS stimulant	*Camellia sinensis*
Camphor	Rubefacient	*Cinnamomum camphora*
Camptothecin	Anticancerous	*Camptotheca acuminata*
(+)-Catechin	Haemostatic	*Potentilla fragarioides*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Convallatoxin	Cardiotonic	*Convallaria majalis*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*

Danthron	Laxative	*Cassia species*
Demecolcine	Antitumor agent	*Colchicum autumnale*
Deserpidine	Antihypertensive, tranquillizer	*Rauvolfia canescens*
Deslanoside	Cardiotonic	*Digitalis lanata*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Digitalin	Cardiotonic	*Digitalis purpurea*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis purpurea*
Emetine	Amoebicide, emetic	*Cephaelis ipecacuanha*
Ephedrine	Sympathomimetic, antihistamine	*Ephedra sinica*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Galanthamine	Cholinesterase inhibitor	*Lycoris squamigera*
Gitalin	Cardiotonic	*Digitalis purpurea*
Glaucarubin	Amoebicide	*Simarouba glauca*
Glaucine	Antitussive	*Glaucium flavum*
Glasiovine	Antidepressant	*Octea glaziovii*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*
Gossypol	Male contraceptive	*Gossypium species*
Hemsleyadin	Bacillary dysentery	*Hemsleya amabilis*
Hesperidin	Capillary fragility	*Citrus species*

Hydrastine	Hemostatic, astringent	*Hydrastis canadensis*
Hyoscyamine	Anticholinergic	*Hyoscyamus niger*
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Kaibic acud	Ascaricide	*Digenea simplex*
Kawain	Tranquillizer	*Piper methysticum*
Kheltin	Bronchodilator	*Ammi visaga*
Lanatosides A, B, C	Cardiotonic	*Digitalis lanata*
Lapachol	Anticancer, antitumor	*Tabebuia sp.*
a-Lobeline	Smoking deterrant, respiratory stimulant	*Lobelia inflata*
Menthol	Rubefacient	*Mentha species*
Methyl salicylate	Rubefacient	*Gaultheria procumbens*
Monocrotaline	Antitumor agent (topical)	*Crotalaria sessiliflora*
Morphine	Analgesic	*Papaver somniferum*
Neoandrographolide	Dysentery	*Andrographis paniculata*
Nicotine	Insecticide	*Nicotiana tabacum*
Nordihydroguaiaretic acid	Antioxidant	*Larrea divaricata*
Noscapine	Antitussive	*Papaver somniferum*
Ouabain	Cardiotonic	*Strophanthus gratus*
Pachycarpine	Oxytocic	*Sophora pschycarpa*
Palmatine	Antipyretic, detoxicant	*Coptis japonica*
Papain	Proteolytic, mucolytic	*Carica papaya*

Papavarine	Smooth muscle relaxant	*Papaver somniferum*
Phyllodulcin	Sweetner	*Hydrangea macrophylla*
Physostigmine	Cholinesterase Inhibitor	*Physostigma venenosum*
Picrotoxin	Analeptic	*Anamirta cocculus*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Pinitol	Expectorant	*Several plants*
Podophyllotoxin	Antitumor anticancer agent	*Podophyllum peltatum*
Protoveratrines A, B	Antihypertensives	*Veratrum album*
Pseudoephredrine*	Sympathomimetic	*Ephedra sinica*
Pseudoephedrine, nor-	Sympathomimetic	*Ephedra sinica*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Qulsqualic acid	Anthelmintic	*Quisqualis indica*
Rescinnamine	Antihypertensive, tranquillizer	*Rauvolfia serpentina*
Reserpine	Antihypertensive, tranquillizer	*Rauvolfia serpentina*
Rhomitoxin	Antihypertensive, tranquillizer	*Rhododendron molle*
Rorifone	Antitussive	*Rorippa indica*
Rotenone	Piscicide, Insecticide	*Lonchocarpus nicou*
Rotundine	Analagesic, sedative, traquillizer	*Stephania sinica*
Rutin	Capillary fragility	*Citrus species*
Salicin	Analgesic	*Salix alba*

Sanguinarine	Dental plaque inhibitor	*Sanguinaria canadensis*
Santonin	Ascaricide	*Artemisia maritma*
Scillarin A	Cardiotonic	*Urginea maritima*
Scopolamine	Sedative	*Datura species*
Sennosides A, B	Laxative	*Cassia species*
Silymarin	Antihepatotoxic	*Silybum marianum*
Sparteine	Oxytocic	*Cytisus scoparius*
Stevioside	Sweetner	*Stevia rebaudiana*
Strychnine	CNS stimulant	*Strychnos nux-vomica*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
a-Tetrahydrocannabinol(THC)	Antiemetic, decrease occular tension	*Cannabis sativa*
Tetrahydropalmatine	Analgesic, sedative, traquillizer	*Corydalis ambigua*
Tetrandrine	Antihypertensive	*Stephania tetrandra*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*
Theophylline	Diuretic, brochodilator	*Theobroma cacao and others*
Thymol	Antifungal (topical)	*Thymus vulgaris*
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata*
Trichosanthin	Abortifacient	*Trichosanthes kirilowii*
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
Valapotriates	Sedative	*Valeriana officinalis*

Vasicine	Cerebral stimulant	***Vinca minor***
Vinblastine	Antitumor, Antileukemic agent	***Catharanthus roseus***
Vincristine	Antitumor, Antileukemic agent	***Catharanthus roseus***

2.5 Role of natural products in tuberculosis chemotherapy

Natural products or their semi-synthetic derivatives currently play important roles in the chemotherapy of tuberculosis. Natural products represent one avenue in the search for new antitubercular agents, with several researchers undertaking screening of natural product extracts as the preliminary step to find new lead compounds (Copp, 2003).

Aminoglycoside antibiotics e.g. streptomycin isolated from *Streptomyces griseus*, (Kuehl and Flynn, 1947), the related compound kanamycin (Cron et al., 1958), and the semi-synthetic analogue amikacin (Kawaguchi et al., 1972), cyclic peptides, e.g. capreomycin, isolated from *Streotomyces capreolus* NRRL 2772 (Herr and Redstone, 1966) are used in combination with other antituberculars as either front-line or second line drugs. These natural products exhibit wide-ranging *in vitro* potencies against MTB, with minimum inhibitory concentrations (MIC) of 0.5µg/ml (streptomycin), 1µg/ml (amikacin), 5µg/ml (capreomycins), 6µg/ml (kanamycin) (Barry et al., 2000).

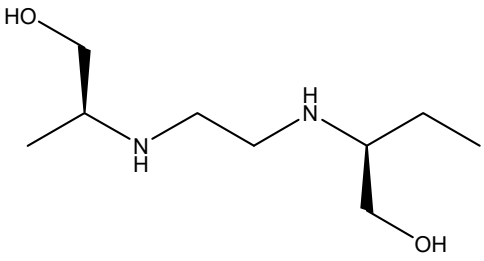
However, despite a decade of research no new selective anti-TB compounds have been identified for the past 30 years. A number of plant extracts with significant anti-tuberculosis activity have been reported in the literature. The past decade has witnessed a

surge in the number of compounds that have been described in the scientific literature as possible antimycobacterial agents. Naturally occurring, pure compounds, as well as extracts from microorganisms and marine organisms have indicated that inhibitory activity against *M. tuberculosis* is widespread (Okunade et al., 2004).

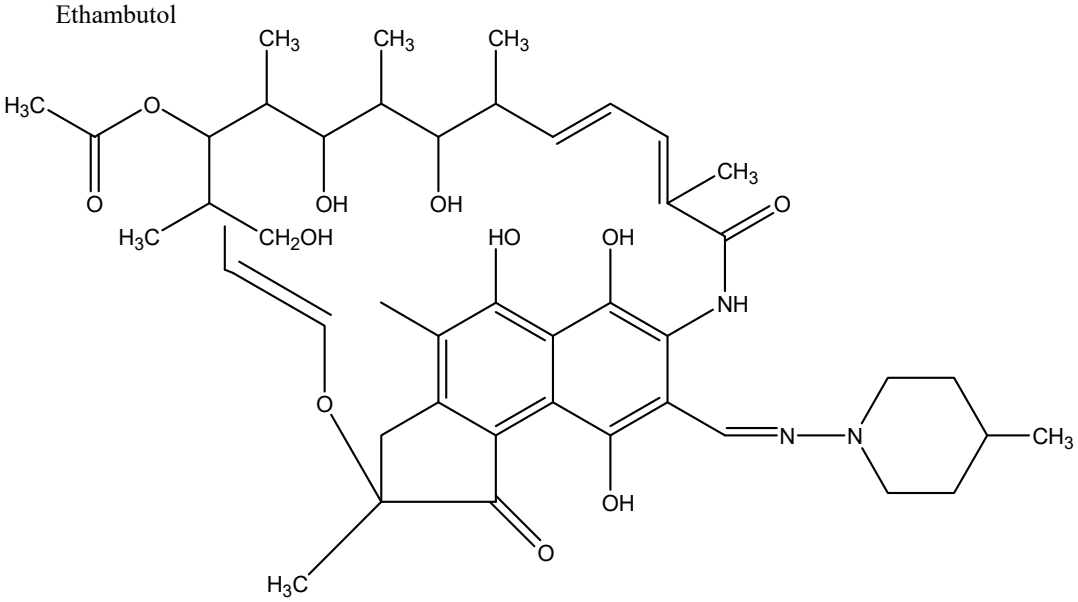
In South Africa it has been reported that people smoke the dried flowers and seeds of *Helichrysumm krausii* to treat pulmonary tuberculosis/infections (Watt and Breyer-Brandwijk, 1962).Worldwide, the 25 most widely used plant species against TB are: viz., *Acorus calamus, Adhatoda vasica, Allium sativum, Alpinia galanga, Artocarpus lakoocha, Caesalpinia pulcherrima, Calotropis gigantea, Canscora decussata, Cinnamomum camphora, Cissampelos pareira, Citrullus colocynthis, Erythrina variegata, Glycyrrhiza glabra, Inula racemosa, Juniperus excelsa, Morinda citrifolia, Ocimum sanctum, Piper cubeba, Plantago major, Portulaca oleracea, Psoralea corylifoila, Sassurea lappa, Solanum dulcamara, Tinospora cordifolia, Zingiber officinale,* which are widely distributed in India from the tropics to alpine Himalayas (Gautam et al., 2007). All these plant species exhibit significant *in vitro* antimycobacterial activity (extracts showed MIC values ranging from 10 to 100µg/ml) and from 11 of these species, active compounds have been isolated with MIC values ranging from 1 to 50µg/ml.

The diterpenoid, '12 demthlymulticauline' isolated from the roots of *Salvia multicaulis*, was found to be more active than ethambutol and as active as rifampin *in vitro* (Cantrell et al., 1999).

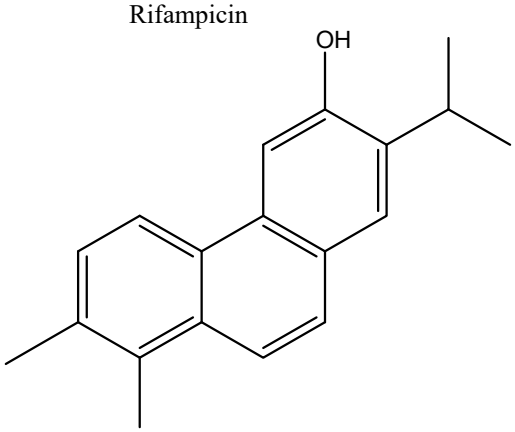
Lakoochin **A** and **B** (stilbene derivatives) isolated from *Artocarpus lakoocha* exhibited MIC values of 12.5 and 50µg/ml, respectively, against MTB. 6β-Benzoyl-7β-hydroxyvouacapen-5α-ol and 6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol isolated from *Caesalpinia pulcherrima* showed MIC values of 25 and 6.25µg/ml, respectively. A fraction containing 1,3,5-trioxygenated xanthone, 1,3,5,6-tetraoxygenated xanthone, and 1,3,5,6,7-pentaoxygenated xanthone isolated from *Canscora decussata* exhibited an MIC value of 10µg/ml. Indicanine B, a coumarin isolated from *Erythrina variegata* had an MIC value of 18.5µg/ml, while licoisoflavone isolated from *Glycyrrhiza glabra* had an MIC value of 25µg/ml. Active compounds, alantolactone and isoalantolactone (sesquiterpene lactones) isolated from *Inula racemosa* exhibited an MIC value of 32 µg/ml. The active compounds of *Juniperus excelsa* are the diterpenes, juniperexelsic acid (MIC, 14.4 µg/ml), sandracopimaric acid (15.0µg/ml), sclareol (6.0µg/ml), and feruginol (5µg/ml). 4-(α-L-rhamnosyloxy) benzyl isothiocynate (MIC 17.5µg/ml) has been identified as the active compound from *Moringa oleifera*. Bakuchiol, a phenolic monoterpene isolated from *Psoralea corylifolia* exhibited an IC_{50} value of 15.79µg/ml. The active compound identified from *Sassurea lappa* was found to be a sesquiterpene lactone, dehydrocostus lactone (MIC, 2µg/ml).



Ethambutol



Rifampicin



12-demethylmulticauline

Figure 2.2 Chemical structures of ethambutol, rifampin and 12 demthlymulticauline

From *Zingiber officinale*, 10-gingerol (MIC 25µg/ml) and 8-gingerol (MIC 50µg/ml) were identified as active compounds. Thus, the active extracts of these 25 species may prove to be useful agents for treatment of TB and the isolated active compounds may be considered as lead compounds for new anti-TB drug development (Gautam et al., 2007).

The 1,4-Naphthoquinone structure is common in various natural products and is found to exhibit a wide range of pharmacological properties. Naphthoquinones have been found to possess antibacterial, antiviral, trypanocidal, anticancer, antimalarial and antifungal activities, as stated by Mital et al, in 2008). Naphthoquinones and their derivatives bearing heterocyclic rings may possibly serve as leads for anti-tubercular activity. The anti-bacterial activity of naphthoquinones is attributed to their aromatic stability. Two napthoquinones, diospyrin and 7-methyljuglone isolated from the *Euclea natalensis* by Lall et al. in 1999 and 2000, exhibited intracellular and extracellular inhibition of MTB comparable to streptomycin and ethambutol (Lall, 1999; Lall, 2000). However, the exact mechanism of action of naphthoquinones in MTB, is not clearly understood. Table 2.4 describes the possible mode of action of naphthoquinones (van der Kooy, 2007).

2.6 Naphthoquinones

Plants produce a large and diverse array of organic compounds known as secondary metabolites or natural products that have no direct function in growth and development, as mentioned earlier in this chapter (Taiz and Zeiger, 2002). Secondary metabolites have been known to play a role in structural support (e.g. lignins) or pigmentation (e.g.

anthocyanins) or are used by plants to defend themselves against herbivores and pathogenic microbes. The three classes of important secondary metabolites are : terpenes – consisting of isopentane units (5-carbon elements), nitrogen-containing compounds – e.g. alkaloids like caffeine, found in coffee and phenolics (Figure 2.3) -containing a hydroxyl functional group on an aromatic ring (Van der Kooy, 2007).

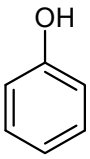


Figure 2.3: Structure of phenolics - containing a hydroxyl functional group on an aromatic ring

The compounds investigated in this study i.e. neodiospyrin, diospyrin and 7-methyljuglone belong to the phenolic group and are known as naphthoquinones (Figure 2.4). Naphthoquinones are secondary metabolites present in several families of higher plants and are widely recognized for their diverse and wide range of pharmacological activities against aerobic and anaerobic bacterial species (Pereira, 2006). In a study by Tokunaga *et al.* (2004), it was reported that naphthoquinones have strong anti-feedant properties and are accumulated by carnivorous plants as defense mechanism against predators. Much of the medicinal functions and potency of the above-mentioned naphthoquinones are due to the aromaticity of the naphthalene ring (Van der Kooy et al., 2007).

In a structure-activity relationship study, (Van der Kooy et al., 2006) showed that the ketone groups on the C1 and C4 atoms are important for antimycobacterial activity. The activity of the compounds of interest in this study, declined dramatically when the ketone was reduced to the corresponding hydroxyl group. The aromaticity of the naphthalene ring also influences potency, with the reduction of the aromatic ring the activity declines sharply.

In 1998, Mallavadhani *et al* described four different biosynthetic pathways for the formation of naphthoquinones:

1) Incorporation of shikimic acid into the benzenoid naphthoquinone ring with retention of the carboxyl group.

2) Homogentisic acid pathway involving the condensation of mevalonic acid and toluhydroquinone.

3) Prenylation of p-hydroxybenzoic acid with geranyl pyrophosphate followed by decarboxylation and ring closure.

4) The polyacetate-melonate pathway.

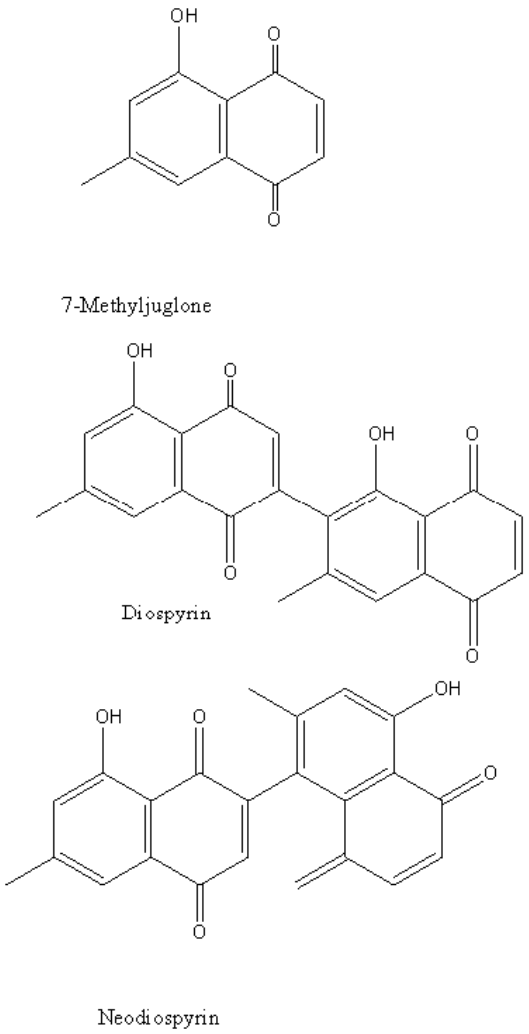


Figure 2.4: a) Diospyrin, Neodiospyrin 7-methyljuglone

For many decades plants containing naphthoquinones have been used for the treatment of various ailments. Due to their molecular structure naphthoquinones confer redox properties, participating in multiple oxidation reactions and serve as vital links in the electron transport chains in metabolic pathways and oxidative processes. The biological

redox cycle of quinones can be initiated by either one or two electron reduction leading to the formation of semiquinones a reaction catalyzed by NADPH-cytochrome P450 reductase. Semiquinones are unstable intermediates that react rapidly with molecular oxygen, and return to their original quinoidal formation thereby generating a superoxide anion radical (O_2^-). Via a superoxide dismutase (SOD)-catalyzed reaction, superoxide can then be converted to hydrogen peroxide (H_2O_2), followed by the formation of a hydroxyl radical (HO^-) by the iron-catalysed reduction of peroxide. In cells, these free radicals are highly reactive species enhancing oxidative stress, and may cause damage to membranes, proteins and also to DNA, which may induce apoptosis and react directly with other cellular macromolecules, such as lipids and proteins (alkylation of crucial proteins and nucleic acids), leading to cell damage (Pinto and Castro, 2009). Naphthoquinones and their derivatives bearing heterocyclic rings may possibly serve as leads for anti-tubercular activity.

Over the past years neodiospyrin, diospyrin and 7-methyljuglone has been closely studied and a wide variety of medicinal functions have been discovered. Diospyrin has been shown to have the following activities: antimalarial (Lall et al., 2005), proapoptotic (Kumar et al., 2009), anti-inflammatory (Hazra et al., 2005), antibacterial (Adeniyi et al., 2000), leishmania inhibitor (Hazra et al, 2002), antimycobacterial (Kuke et al., 1998), and topoisomerase inhibitor (Likhitwitayawuid et al., 1999; Tazi et al., (2005)

7-methyljuglone has been shown to have the following activities: antifungal (Steffen and Peschel, 1975), termicidal (Carter et al., 1978), Ca^{2+}-channel blocking (Neuhaus-Carlisle

et al., 1997), antimicrobial and cytotoxic (Gu et al., 2004), antimycobacterial (Lall et al., 2005), active against ants (Suzuki et al., 1995), anti-feedant activity (Tokunaga et al., 2004) and anti-tumor inhibitory activity (Wube et al., 2005).

Neodiospyrin has been shown to have the following activities: antimycobacterial (Van der Kooy et al., 2006) and tumor inhibitory (Wube et al., 2005)

The mechanism of action of these compounds remains to be investigated. Due to the generation of oxygen radical species, naphthoquinones were often tested for possible anticancer properties (Wang et al., 2003). Diospyrin has shown inhibitory activity for murine tumors *in vivo* and in human cancer cell lines (Hazra, 2005) by binding to electron transport chains (Bailly, 2000) and preventing or reversing formation of the topoisomerase I and DNA complex (Cushion et al., 2000). 7-methyljuglone has been shown to inhibit the protein kinase C which gives the compound antitumor properties (Timothy et al., 1995)

The anti-bacterial activity of naphthoquinones is attributed to their aromatic stability. Two naphthoquinones, diospyrin and 7-methyljuglone isolated from the *Euclea natalensis* by Lall et al., in 1999 and 2000, exhibited intracellular and extracellular inhibition of the growth of MTB, comparable to streptomycin and ethambutol (Lall, 1999; Lall, 2000). 7-methyljuglone is of particular interest with respect to antimycobacterial activity for MTB due to the fact that its structure is very similar to menaquinone which occurs only in MTB. Most bacteria and mammals use ubiquinone to

mediate electron transfer between different membrane bound enzymes of the respiratory chain. MTB lacks ubiquinone and only uses mena quinone in the electron transport chain (Truglio, 2003). Due to structural similarities of 7-methyljuglone and menaquinone it is possible that 7 methyljuglone may interfere with the electron transport in MTB by either interacting with enzymes in the electron transport chain or binding to the menB (1,4-dihydroxy-2-naphthoyl-coenzyme A synthase) enzymes that are responsible for the formation of menaquinone and thereby causing the flow of electrons to cease. As a consequence, ATP production will be disturbed and this may have a detrimental effect on MTB (Van der Kooy et al., 2007).

The exact antimicrobial mechanisms exerted by these compounds are, however, far from established. In this study, the possible mode of action of neodiospyrin, diospyrin and 7-methyljuglone on MTB has been further investigated.

Nitroimidazole analogs, macrolides, pleuromutillins, isocitrate lyase inhibitors, InhA inhibitors and methyltransferase inhibitors are in the preclinical testing stage and are scheduled to be released by 2015. A few promising compounds e.g. moxifloxacin, gatifloxacin, diarylquinoline TMC207, nitroimidazole OPO67683, pyrrole LL3858, nitromidazole Pa-824, diamine SQ-109quinolones and nitroimidazole analogs, are currently in phase II and III clinical trials and are scheduled to be released between the years 2010 and 2012 according to the TB Drug Alliance Consortium (Figure 2.5) (Table 2.4) (http://www.tbalert.org/resources/documents/Freire.pdf).

Table 2.4 The mode of action of naphthoquinones and author references (Van der Kooy, 2007)

Compound	Mode of action	Reference
Diospyrin	Prevent or dissociates the topoisomerase I / DNA complex. Binds to electron transport chain	Bailly, 2000 Cushion et al., 2000
Isodiospyrin	Binds topoisomerase I- preventing it from binding to DNA	Ting et al., 2003
Juglone	Inhibited respiration in bean and lettuce plants and binds to thiol groups of peptides	Li et al., 1993
Plumbagin	Superoxide generator	Wang et al., 1998

Table 2.5 Compounds discovered for TB chemotherapy which are currently in preclinical and clinical stages of development

Compounds, analogs & derivatives (discovery stage)	Preclinical stage	Clinical stage
1.Nitroimidazole analogs **2. Carboxylates &** **Pyrroles** **3.Quinolones** **4. Macrolides** **5.Isocitrate Lyase Inhibitors** **6.Pleuromutillins**	**Nitroimidazole Back-up compound**	**Nitroimidazole PA-824** **Moxifloxacin** **Nitroimidazole OPC-67683**

7.Malate synthase inhibitors **8.Protease inhibitors** **9.Riminophenazines** **10.Capuromycins**	

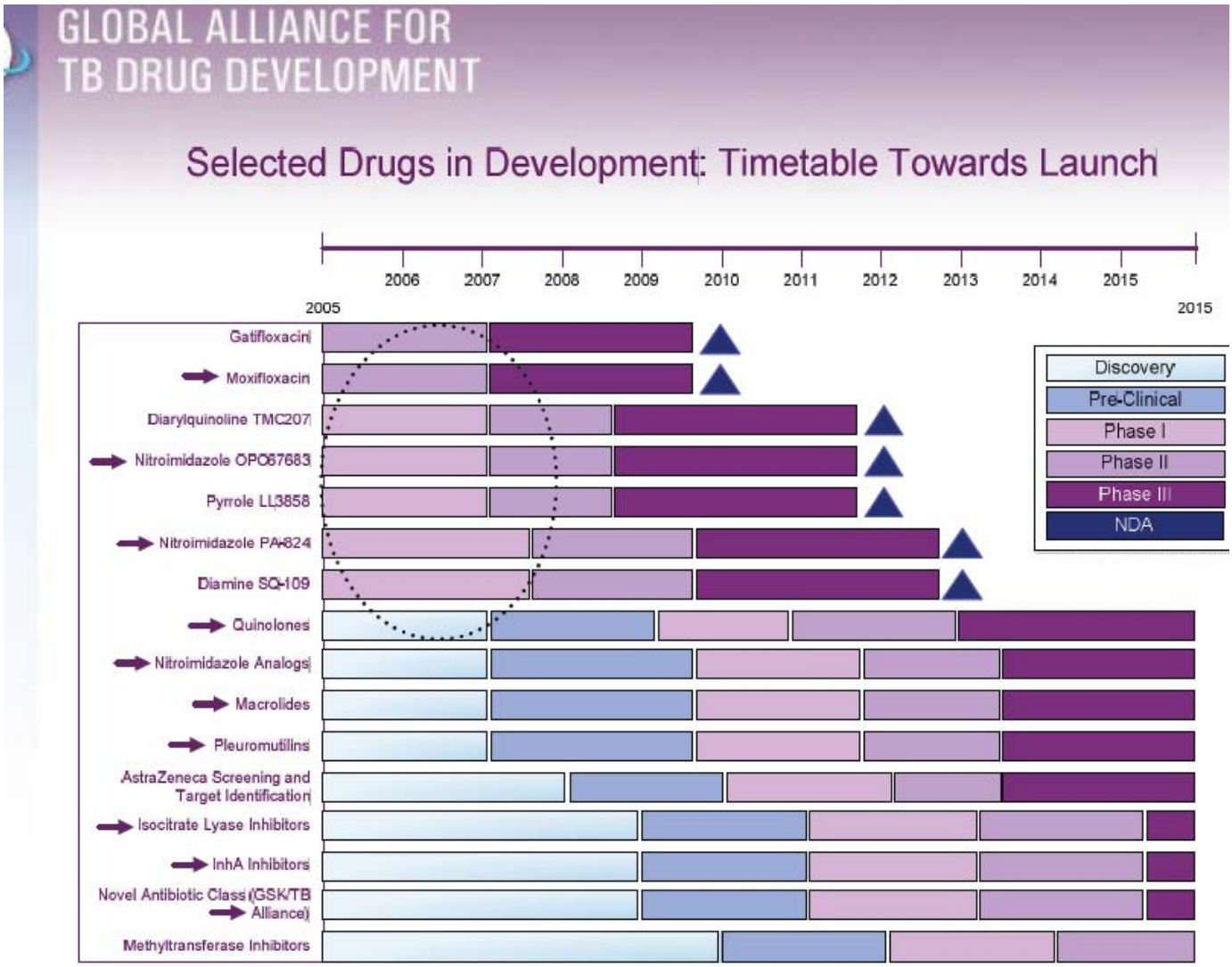


Figure 2.5 Selected compounds from natural products in development stages (http://www.tbalert.org/resources/documents/Freire.pdf)

6. Conclusion

With the current TB treatment regimens necessitating a lengthy 6-9 months duration of treatment with a complex 3-4 drug combination, leading to decreased compliance, MDR-TB on the rise, the recent emergence of XDR-TB, no new class of TB drugs in 30 years, and a decrease in health system efficiencies, there is still an urgent need to identify new TB drugs that are comparable with the standard anti-TB drugs being used today. As there is an apparent correlation between antimycobacterial activity and the activity of traditional plant-derived remedies against TB and TB-related diseases, the search for novel natural product leads in anti-TB drug discovery and development appears justified.

OBJECTIVES OF STUDY

An intensive investigation of South African medicinal plant species traditionally used in the treatment of TB showed that crude extracts from the plant *Euclea natalensis* possess significant activity against MTB. Further studies revealed that the anti-mycobacterial activity was attributable to a bisnaphthoquinoid, diospyrin, and 7-methyljuglone, isolated from the crude extract of *Euclea natalensis* (Lall and Meyer, 2001). Importantly, inhibitory activity was observed with both drug-susceptible and drug-resistant strains of MTB. This study entailed intensive training in isolation procedures and all compounds have been successfully isolated from roots of *Euclea natalensis* and investigated their cytotoxic potential for eukaryotic cells, activities against intracellular MTB, and possible

mode of action against MTB. Also, synthetic 7-methyljuglone was included in this study to compare its activity with that of isolated 7-methyljuglone.

The objectives of the present study are as follows:

1. To isolate bioactive antimycobacterial compounds from the roots of *Euclea natalensis*
2. To evaluate the cytotoxicity of the compounds using human cell lines *in vitro*
3. To investigate their intracellular efficacy against MTB in a human macrophage-like cell line
4. To investigate the possible mode of action of the compounds against MTB, specifically effects on the uptake of K^+ by this microbial pathogen, as well as effects on microbial energy metabolism.
5. To determine the minimum inhibitory concentration of the compounds for *M. smegmatis*

This work was carried out independently by myself, over a period of six years in parallel with other researchers who are also interested in these agents.

3.1. Introduction

Traditional medicine is used by a large number of the South African population for their physical as well as psychological health needs (Rabe and van Staden, 1997). Of particular interest in this study is the plant *Euclea natalensis,* which belongs to the Ebenaceace family and is widespread in the tropics and subtropics. There are 16 Euclea species found in South Africa located mainly in the Eastern Cape and Kwa-Zulu Natal (Jordaan, 2003). *Euclea natalensis* is a shrub or a small to medium sized tree which occurs in a variety of habitats including the coastal and inland forests, as well as the bushveld in Southern Africa (Van Wyk and Van Wyk, 1997).

Figure 3.1 *Euclea natalensis* (www.worldbotanical.com)

Commonly known as Natal Guarri/ Natalghwarri, the roots of *Euclea natalensis* are used by indigenous people of Southern Africa for treating various bacterial infections (Watt and Beyer Brandwijk, 1962) (Figure 3.1). Powdered root bark of this species is used as an ingredient in medicines to treat urinary tract infections, venereal diseases and dismennorrhea. The twigs and roots of *Euclea natalensis* are used to clean teeth and gums (Stander and Van Wyk, 1991). The Zulu South African tribe also uses the bark to treat TB-related symptoms such as chest diseases, bronchitis, pleurisy and asthma.

Several secondary metabolites e.g. dihydroxyursanoic acid and triterpenoids have been isolated from *Euclea natalensis* (van der Vijer and Gerritsma, 1974; Ferriera et al., 1997; Lall and Meyer, 2001). Two known naphthoquinones were isolated previously, diospyrin and 7-methyljuglone from the roots of *Euclea natalensis* A. DC (Lall and Meyer, 2000). When tested *in vitro*, these naphthoquinones showed potent antituberculosis activity against drug-resistant TB. In order to further investigate the mechanism of action of these compounds and their intracellular bioactivities in human-macrophages infected with *M. tuberculosis*, these naphthoquinones had to be first isolated from the roots of the *Euclea natalensis.* Over the years, various authors used chloroform extracts of different plant parts (Ferreira et al., 1973; Van der Viyver and Gerritsma., 1974) to extract naphthoquinones from various plant species. In the current study, the chloroform extract of the roots of *Euclea natalensis* was also used to isolate naphthoquinones.

This chapter deals primarily with the isolation of the naphthoquinones (diospyrin and neodiospyrin) using various chromatographies such as thin layer chromatography,

column chromatography and high performance liquid chromatography. The naphthoquinones isolated were used in the studies described in the subsequent chapters to test the activities of the compounds in human-macrophages infected with the H37Rv strain of *M. tuberculosis*, as well as to probe as possible mechanisms of antimycobacterial action.

3.2 Materials and methods

3.2.1 Materials

Euclea natalensis A.DC. (Ebenaceae) root material was collected in the KwaZulu-Natal province of South Africa. The samples (N.L.22) were deposited at the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria.

3.2.2 Methods

3.2.2.1 Isolation of active compounds

In previous studies conducted by Van Der Kooy et al. (2007), it was shown that the concentration of diospyrin was higher in the root bark than in the inner root of the *E. natalensis*. Therefore to obtain the highest yield, the root bark was used in this study.

Various techniques are available for the purification and identification of natural products. During this study the compounds were purified by means of column

chromatography and compared to authentic standards with the use of thin layer chromatography (TLC) and spectroscopic analysis.

3.2.2.1 .1 Neodiospyrin and diospyrin isolation and purification

Semipure fractions containing both 7-methyljuglone, diospyrin and neodiospyrin were taken from plant science department, and prepared as follows: The fractions (E-11) were subjected to Sephadex LH-20 column (2.0 x 40.0 cm) was prepared and washed with ethanol and eluted with a 100% ethanol (2 L) to give two main fractions. This procedure was repeated on Sephadex columns to give pure diospyrin and neodiospyrin. The diospyrin and neodiospyrin were spotted on a TLC plate (Merck, Kieselgel F_{254}) (solvent system was 9:1 hexane: ethyl acetate) and subjected to UV scrutiny.

3.2.2.1 .2 7-Methyljuglone isolation and purification

A Sephadex LH-20 column (2.0 x 40.0 cm) was prepared and washed with ethanol and the fraction (EN-3) was fractionated with a 100% ethanol the eluting fractions were (55) spotted on a TLC plate (solvent system was 9:1 dichloromethane: methane) and subjected to detection process as given before, according to the TL profiles, fractions 14-33 were pooled and dried using the rotary evaparator. This was rechromatographed over silica gel columns with dichloromethane 0.1% methanol mobile phase and 21 fractions were collected. This procedure was repeated twice to yield pure 7-methyljuglone (1.5 mg). ^{1}H-spectra of the isolated compounds was recorded in $CDCl_3$ on a Varian Mercury-200 spectrometer. The ^{1}H-shifts of compounds matched the previously reported literature

values and confirmed by co-spotting with reference compound available in the department (Vijver and Gerritsma, 1974; Khan et al., 1978).

3.4. Confirming purity of isolated compounds

Diospyrin and neodiospyrin were identified by means of TLC. ^{1}H NMR spectra of all the isolated compounds were recorded in $CDCl_3$ on a Varian Mercury-200 spectrometer. The ^{1}H-shifts of compounds matched the previously reported literature values (Vijver & Gerritsma, 1974, Khan et al., 1978)

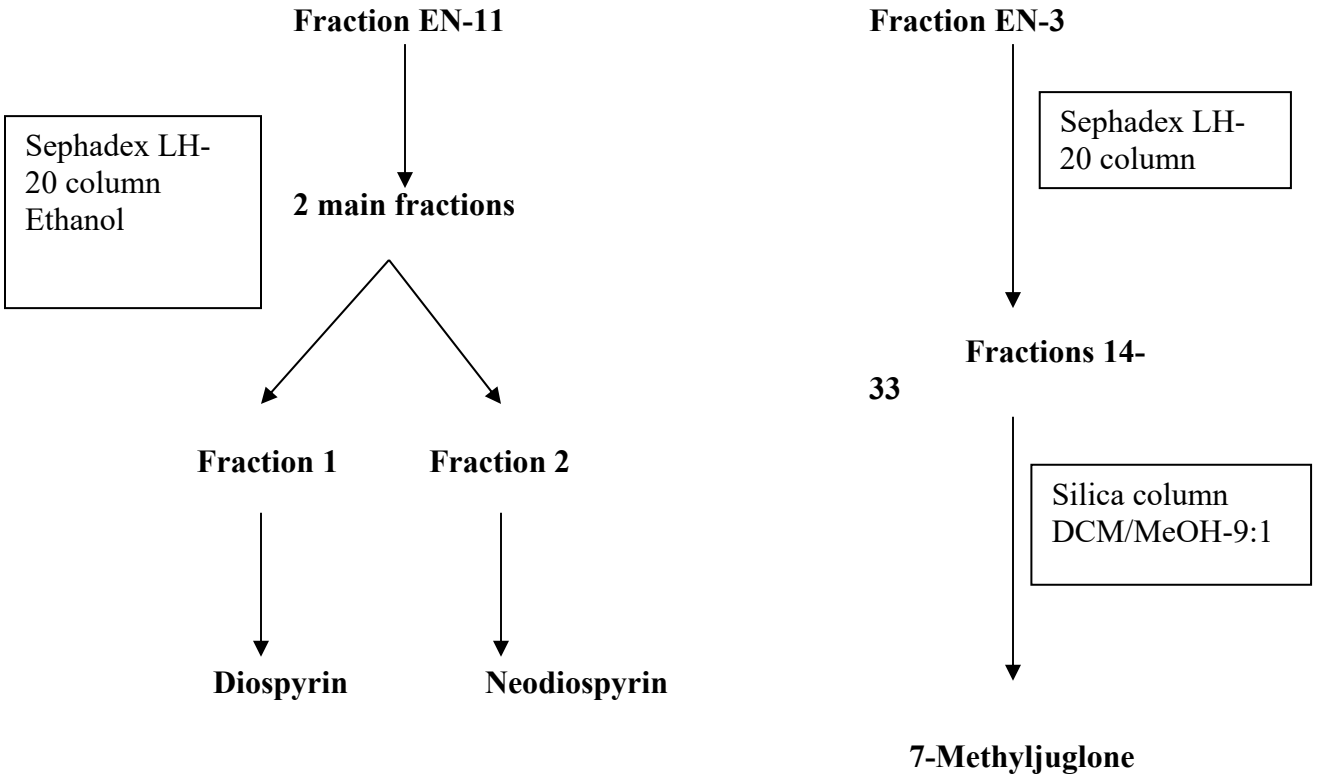


Figure 3.2 The chromatographic steps followed to isolate the three naphthoquinones

3.5 Results

3.5.1 Isolated compounds

Diospyrin, neodiospyrin and 7-methyljuglone were identified by means of TLC in the presence of reference samples. ^{1}H NMR analyses were also performed on these three compounds and the results compared to published data (Vijver and Gerritsma, 1974, Khan et al., 1978; Hazra et al., 1984).

3.5.1.1 ^{1}H NMR analysis of isolated compounds

Diospyrin, neodiospyrin and 7-methyljuglone were identified by ^{1}H NMR and compared to published results (Lillie and Musgrave, 1977; Kumari et al., 1982). The ^{1}H MNR chemical shifts of the isolated naphthoquinones are shown in Table 3.1

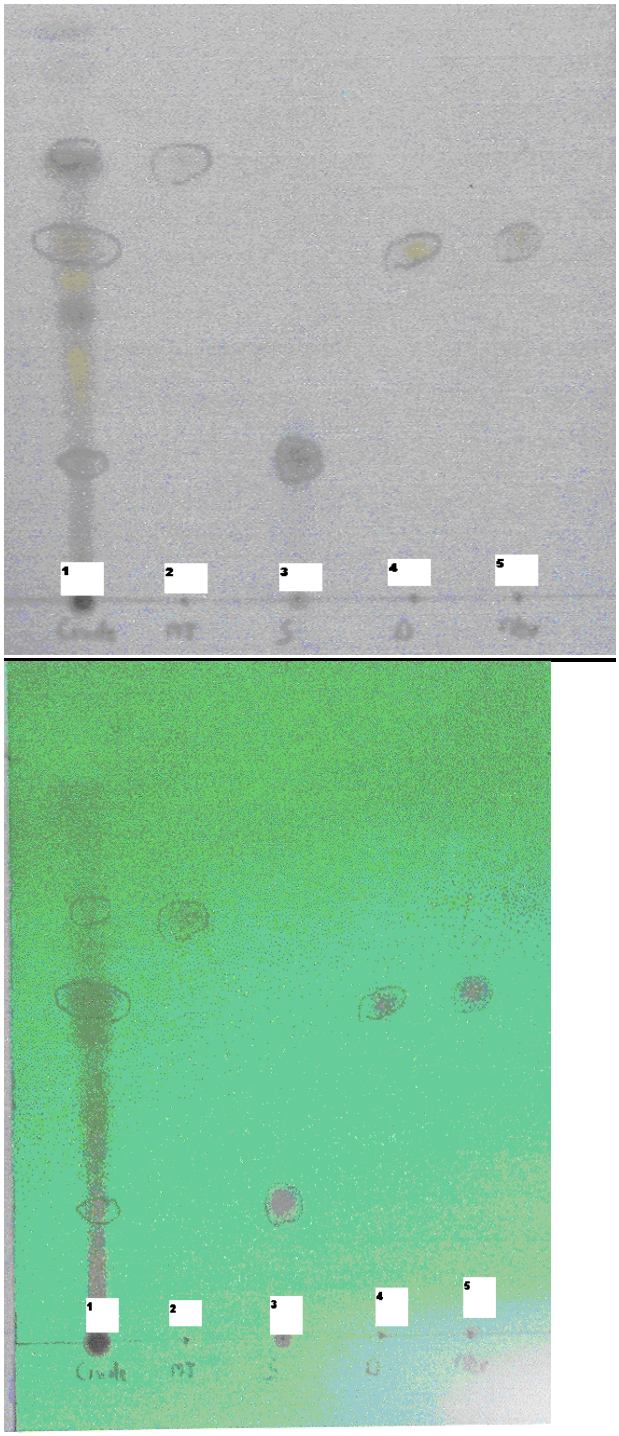


Figure 3.3 TLC profile of the crude extract under visible light after spraying with vanillin reagent (top) and under ultraviolet (UV) light (bottom) Lane 1: crude extract, lane 2: 7-methyljuglone, lane 3: shinanalone, lane 4: diospyrin, lane 5: neodiospyrin.

Table 3.1- NMR data for selected naphthoquinones

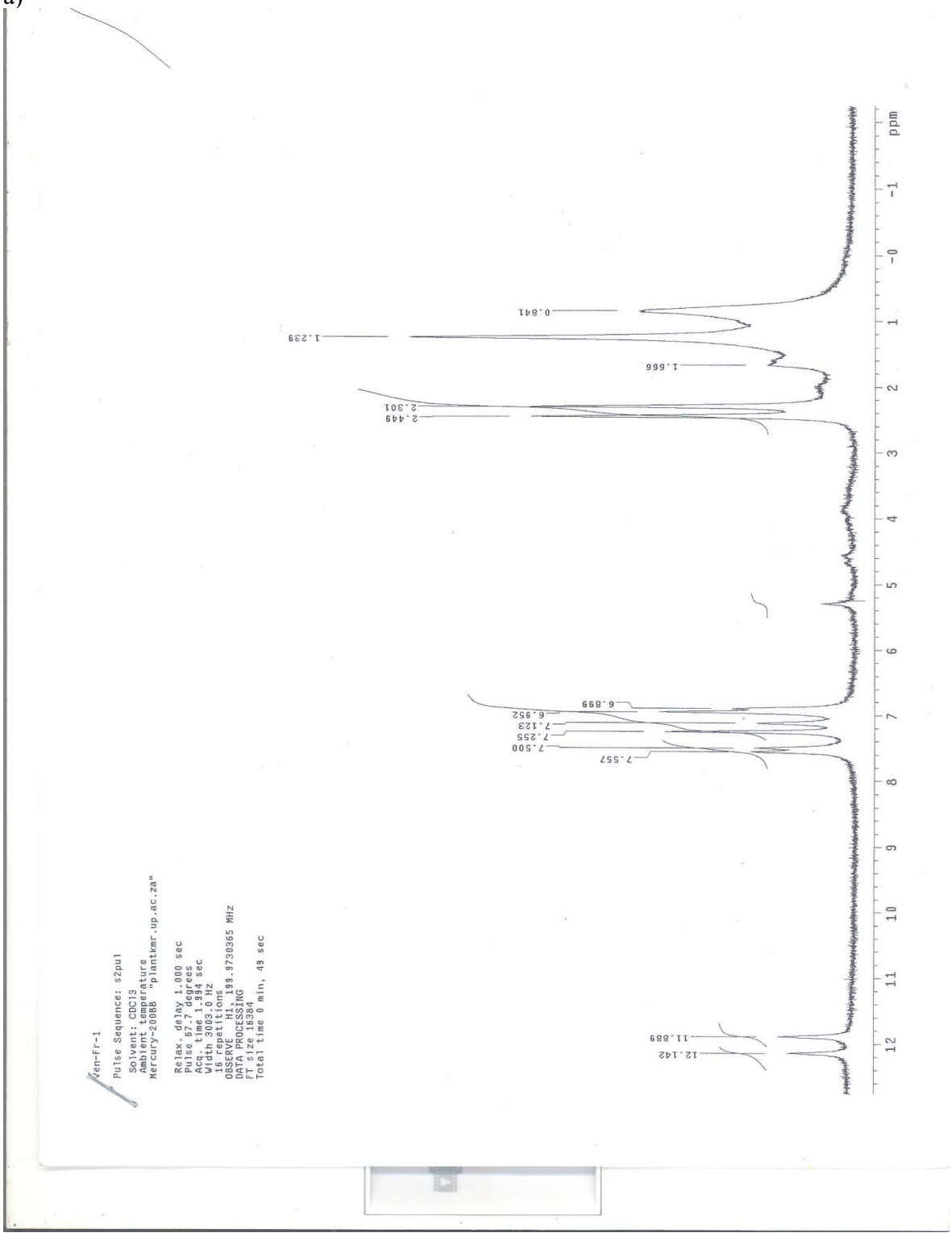
Compound	Unit	Chemical shifts					
		H-2	H-3	H-6	7-Me	H-8	5-OH
Diospyrin	A		6.88	7.11 bd J=1	2.43	7.49 bd J=1	11.85
	B	6.93	6.93		2.29	7.54	12.11
Neodiospyrin	A	6.76 d J=10.35	6.92 d J=10.35	7.25	2.28		12.27
	B	6.6		7.09 bd J=1	2.45	7.52 bd J=1	11.73
7-Methyljuglone	A	6.89	6.89	7.06	2.42	7.43	11.83

d = duplet, bd = broad duplet, mp = multiplet, ,J = coupling constant in Hz.

3.5.1.1.1 Diospyrin

The compound was identified as the binaphthoquinone, diospyrin, by comparison of its ^{1}H-NMR spectral data with published spectra (Hazra et al., 1984). The ^{1}H-NMR spectrum exhibited signals at δ 2.28 and δ 2.43 from the two methyl groups, at δ 11.86 and δ 12.11 from the two hydroxyl groups, proton singlets at δ 6.89, 7.10, 7.48, 7.54 and a doublet at δ 6.93 (See Figure 3.3).

a)

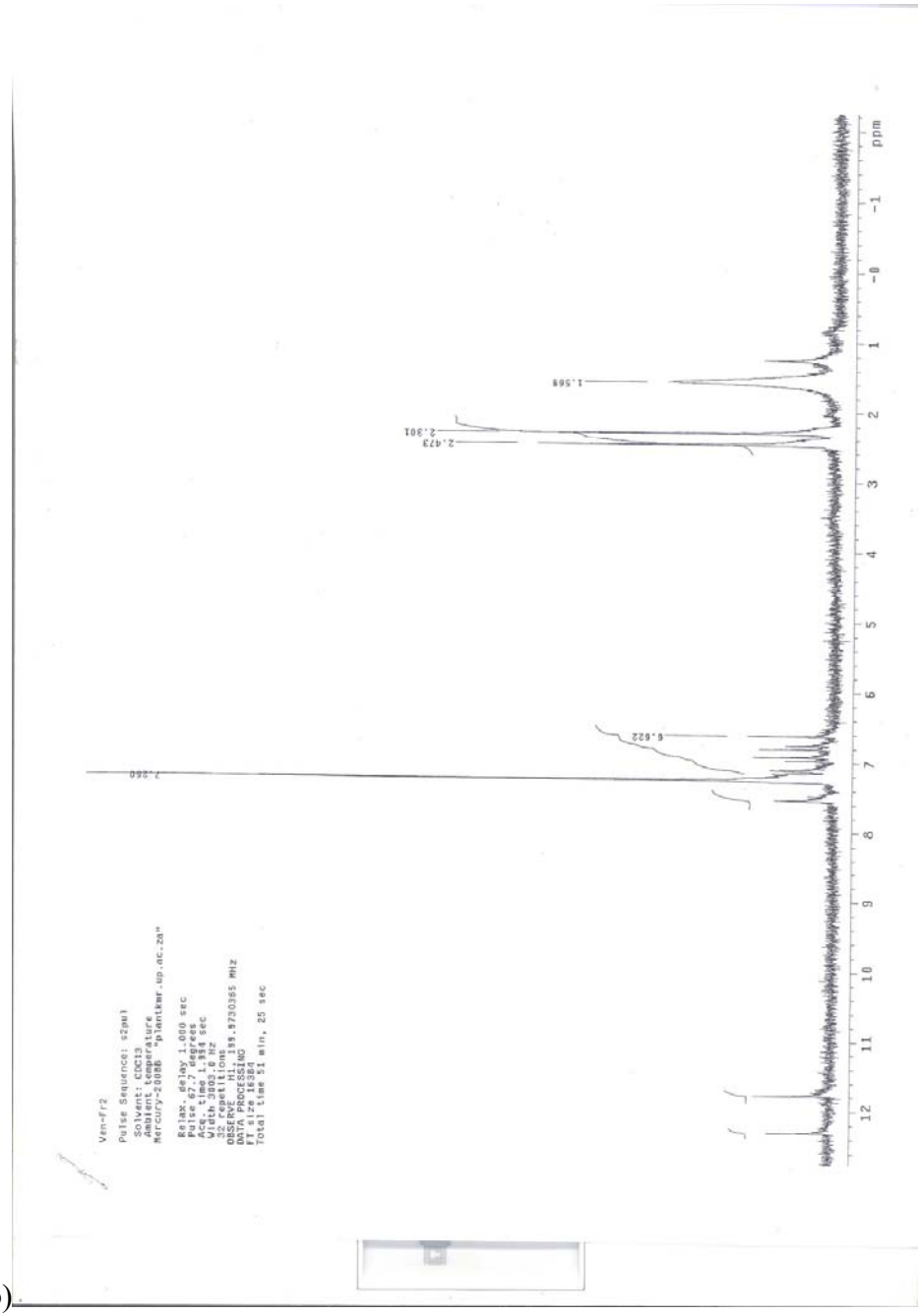


3.51.1.2 Neodiospyrin

The compound was identified as the binaphthoquinone, diospyrin, by comparison of its ^{1}H-NMR and ^{13}C-NMR spectral data with published spectra (Van der Kooy, 2007). The ^{1}H-NMR spectrum exhibited signals at δ 2.28 and δ 2.45 from the two methyl groups, at δ 11.73 and δ 12.27 from the two hydroxyl groups, proton signals at δ 6.76 (d, J=10.4, H-2), 6.70 (s, H-2'), 6.92 (d, J=10.4, H-3), 7.25 (s, H-6), 7.09 (bd, J=~1, H-6'), 7.52 (bd, J=~1, H-8), (Figure 3.4).

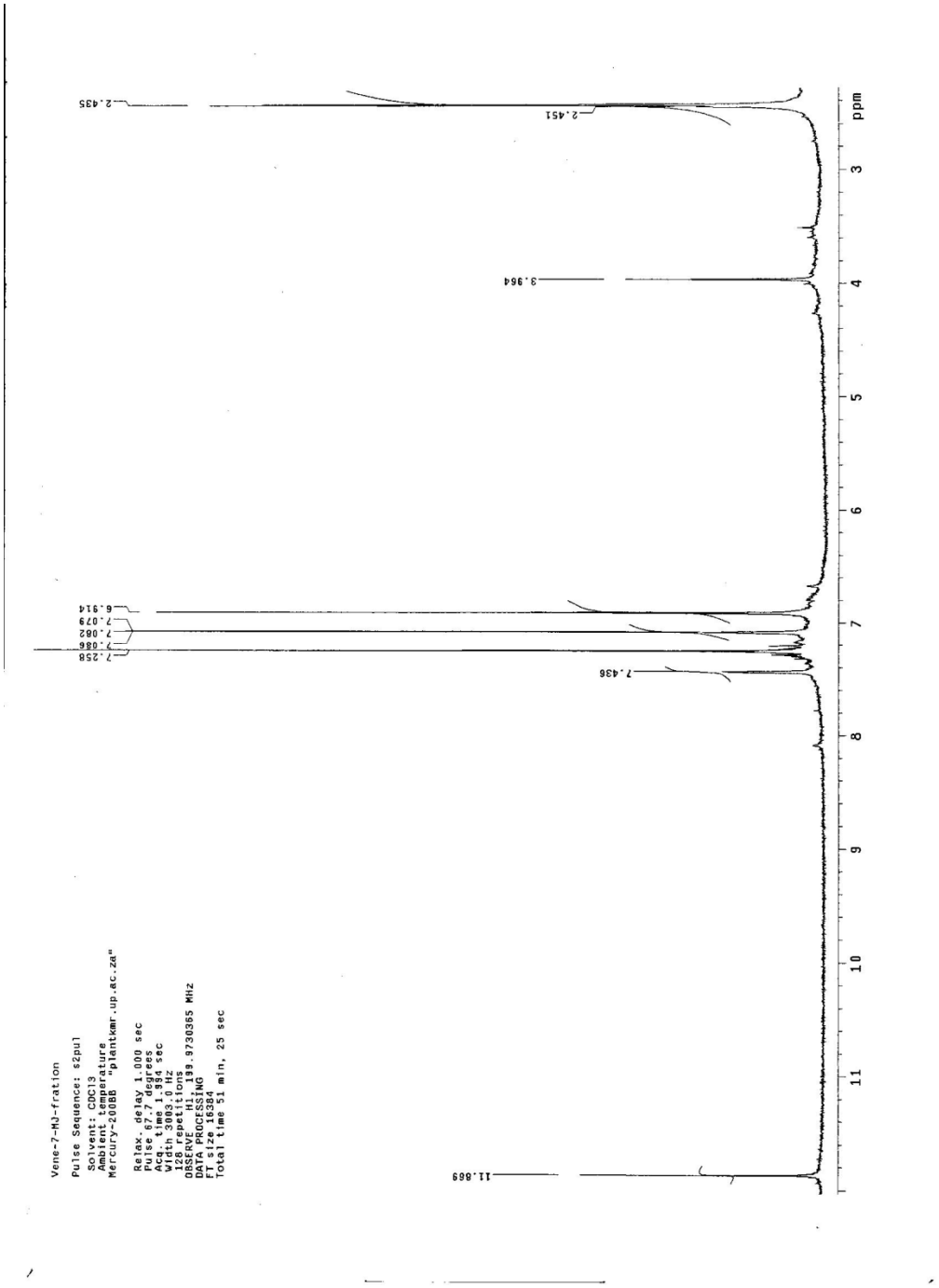
The identification was confirmed by direct comparison with authentic samples on TLC. Neodiospyrin has also been also isolated from the roots of *Diospyros kaki, D rotundifolia* and wood and fruit of *D. Ismaili.*

b)



3.5.1.1.3 7-Methyljuglone

Synthesised 7-methyljuglone was authenticated by means of ^{1}H-NMR analysis. The ^{1}H-NMR data for 7-methyljuglone are δ 2.37 (s, 3H, 7-Me); 6.88 (s, 2H, 2, 3 H); 7.06 (d, J=0.54, 1H, 8H); 7.42 (d J=0.54, 1H, 6H); 11.83 (s, 1 H, 5-OH). (See Figure 3.4c).



c)

Figure 3.4:^{1}H NMR (300 MHz, $CDCl_3$) spectra of a) neodiospyrin, b) diospyrin and c) synthetic 7-methyljuglone

Diospyrin, neodiospyrin and 7-methyljuglone were identified by ^{1}H NMR and compared to published results (Lillie and Musgrave, 1977; Kumari et al., 1982).

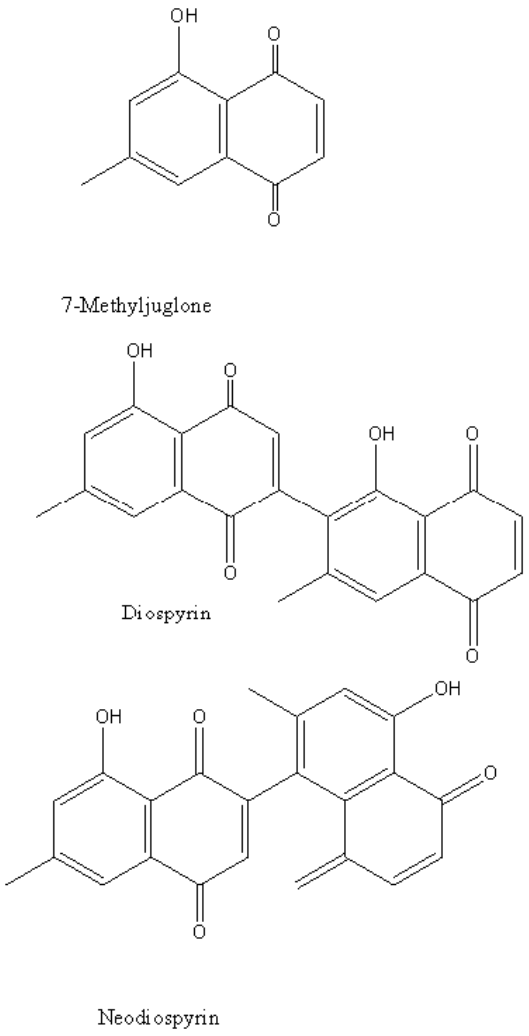


Figure 3.5 Chemical structure of the three naphthoquinones isolated

3.6 Discussion

With approximately 3 million deaths associated with TB annually there is an urgent need to discover new antimycobacterial compounds, particularly those effective against TB infection (Smith et al, 2004). Over the years, natural products with diverse biological activity have contributed to the development of nearly 75% of anticancers agents in modern Pharmacia and 50% of prescription drugs in the United States have natural product origin (Grifco et al, 1995; Tan, 2002).

In this study, the chloroform extract of the dried roots of *Euclea natalensis*, belonging to the family *Ebenacae*, was used to isolate the naphthoquinones. The two compounds used in this study were previously isolated by other researchers.

In 2006, Van der Kooy et al., isolated neodiospyrin from the roots of *Euclea natalensis.* In 1961 Kapil and Dhar were the first to isolate diospyrin from *Diospyros montana* found in India. 7- Methyljuglone was isolated from the dried roots of *Diospyros Ferrea* by Tezuka et al., in 1973 and also by Van der Vijver and Gerritsma in 1974 from the roots of *Euclea natalensis.*

Isolation and purification of diospyrin and neodiospyrin, although previously undertaken by others, proved to be a challenging and time-consuming assignment, but was nevertheless an essential prerequisite for the subsequent studies described in this thesis.

4.1 Introduction

During the past decades, medicinal plants have been intensively studied in terms of whether their traditional uses are supported by actual therapeutic effects, or are merely based on folklore (Locher et al., 1995; Jager et al., 1996; Cunningham, 1998). Recently, a number of compounds have been identified from traditional remedies that may have potential antituberculosis activity. More and more people tend to accept traditional medicine as an alternate form of healthcare. However, crude extracts of these remedies, as well as putative purified active ingredients, must be tested for non-specific cytotoxicity using normal human cells (Rabe, 1997). *In vitro* cytotoxicity tests are based on the idea of basal toxicity i.e. toxic chemicals affect the basic functions of the cell and thus toxicity can be measured by assessing cellular damage (Roper and Drewinko, 1976). Cytotoxicity assays measure 3 basic parameters i.e.:

a) Measurement of cellular metabolic activity
b) Measurement of membrane integrity and
c) Direct measure of cell number

In this chapter, the cytotoxic potential of three compounds diospyrin, synthesized 7-methyljuglone and neodiospyrin on a human macrophage cell line and African green monkey kidney cells are reported.

4.2 Materials and methods

4.2.1 Cell culture

4.2.1.1 Materials

Dulbecco's Eagles's Minimum Essential Medium with Earle's salts, L-glutamine and $NaHCO_3$ (MEM), trypsin-EDTA, and trypan blue were supplied by the Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated fetal calf serum (FCS), sterile cell culture flasks and plates were purchased from Sterilab Services (Kempton Park, Johannesburg, SA). Phosphate-buffered saline (PBS), penicillin, streptomycin and fungizone were obtained from Highveld Biological (Sandringham, SA). All other chemicals were of analytical grade and supplied by the Sigma Chemical Co. (St. Louis, MO, USA).

4.2.1.2 Cell cultures

Highveld Biological (Sandringham, SA) supplied the THP-1 cell line which is a continuous cell culture line of lung macrophages. Vero cells (African green monkey kidney cells) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown as monolayers in Dulbecco's Eagle's minimum essential medium (MEM) (see Appendix A) at 37°C in a humidified atmosphere

containing 5% CO_2 in MEM supplemented with 10% heat-inactivated FCS, penicillin (100µg/l), streptomycin (100µg/l) and fungizone (250µg/l).

4.3. Preparation of synthesized 7-methyljuglone, diospyrin and neodiospyrin stock solutions

Synthesised 7-methyljuglone, diospyrin and neodiospyrin were dissolved in dimethyl sulphoxide (DMSO) to give a final concentration of 50mg/ml for each agent. These stock solutions were diluted with medium to the desired concentrations for the experiments. The appropriate concentrations of DMSO were included in all control systems and never exceeded 0.1% (v/v). The compounds were freshly prepared for each experiment conducted in this study, due to their instability when frozen. The compounds were also sonicated to ensure that they were completely dissolved. Synthetic 7-methyljuglone was provided by Dr. Anita Mahapatra.

4.4 Cytotoxicity assays

4.4.1 Materials

Cytotoxicity was measured using the XTT (sodium 3'-[1-(phenyl amino-carbonyl)-3,4 tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) Cell Proliferation Kit II (Roche Diagnostics, Johannesburg, South Africa). The XTT assay can be used for the same applications as the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) assay. The assay is based on the conversion of the yellow tetrazolium salt XTT into an orange formazan dye by metabolically active cells. In contrast to the purple formazan crystals, which are formed in the MTT assay, the XTT formazan dye is soluble in aqueous solutions, and can thus be directly quantitated with an ELISA reader without the need for a solubilization step.

4.4.2. Methods

THP-1 cells and Vero cells were maintained in culture flasks in complete Minimum essential Medium, Eagle and subcultured when a confluent monolayer was reached. Cells were trypsinised (0.25% trypsin containing 0.01% EDTA) for 10 min at 37 ºC and trypsinisation was stopped using complete medium. Cells were seeded into the wells of microtitre plates at a density of 1 X 10^5 viable cells per well. After 24h, dilution series were made of the compounds (0.3µg/ml-50µg/ml (Vero cells) and 3.125µg/ml-50µg/ml (THP-1 cells)) and added to the cells in the wells of the microtitre plates and incubated for 72h at 37°C. Zearalanone (1µM final, Sigma) was used as a positive control. Cell proliferation was measured using the XTT cell proliferation assay kit (Roche Diagnostics, Johannesburg, South Africa). Five ml of XTT labelling reagent and 0.1ml electron coupling reagent were mixed together and 30µl was added via pipette into the wells of 96-well plates.The experiment was terminated by replacing the growth medium with 300µl of 1% glutaraldehyde in PBS for 15 minutes. After incubation the absorbance of the samples was measured at 490nm using an ELISA plate reader (EL$_X$800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden, SA)) with a reference wavelength set at 690nm. Results shown are representative of three independent experiments (each conducted in triplicate).

4.5 Statistical analysis of data

Statistical analysis of the data was conducted as discussed with Dr. P. Becker of the Unit for Biostatistics at the MRC. As mentioned above, experiments were performed at least three times and three replicate wells were used per treatment. The significance of differences between untreated controls and treated test were analyzed using the Students' paired t-test. Differences with a *p*-value of < 0.05 were considered to be statistically significant.

4.6 Results

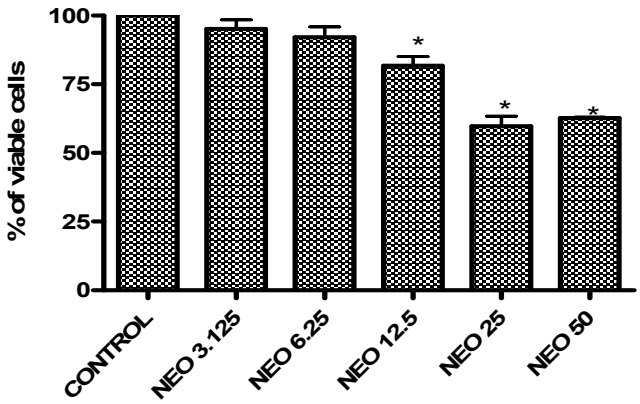
4.6.1 Effects of synthetic 7-methyljuglone, neodiospyrin and diospyrin on the viability of THP-1cells

The effects of the test compounds on the survival of THP-1 cells are shown in Figure 4.1a,b and c. At concentrations between 3.125µg/ml and 50µg/ml ($p < 0.005$) cell growth was inhibited in a dose-related fashion, in the case of all three experimental compounds, achieving statistical significance at a concentration of 12.5µg/ml in the case of all three naphthoquinones. The IC_{50} values for all three test naphthoquinones for both cell lines are showm in Table 4.1.

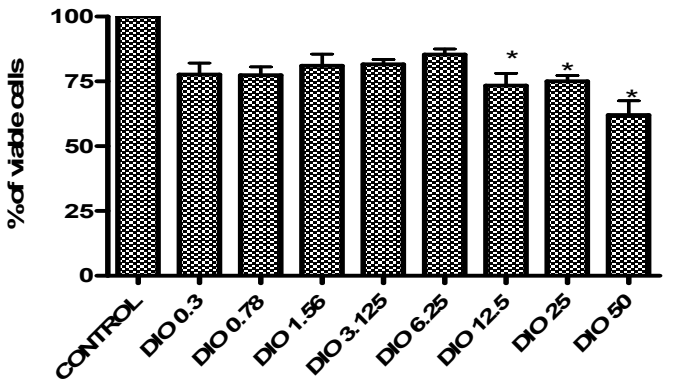
4.6.2 The 50% inhibitory concentration (IC_{50}) of the test compounds for VERO and THP-1 cells

Table 4.1 IC_{50} values of the synthetic 7-methyljuglone, neodiospyrin and diospyrin compounds for VERO and THP-1 cells

Compound	IC_{50} VERO CELLS	IC $_{50}$ THP-1 CELLS
Synthetic 7-methyljuglone	<50 µg/ml	50 µg/ml
Neodiospyrin	50 µg/ml	>50 µg/ml
Diospyrin	50µg/ml	>50 µg/ml
INH	> 200µg/ml	>200µg/ml



a)



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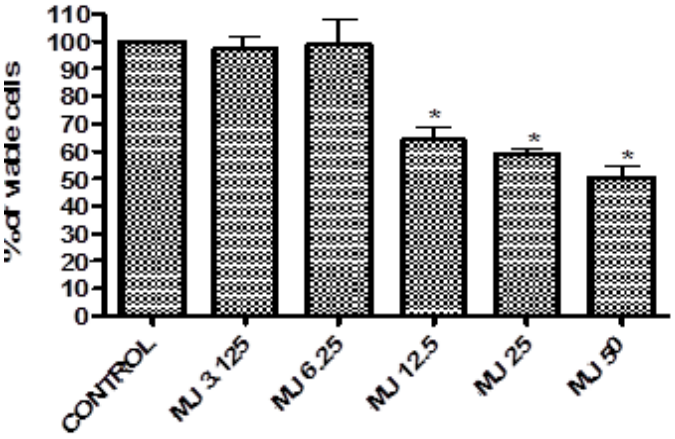


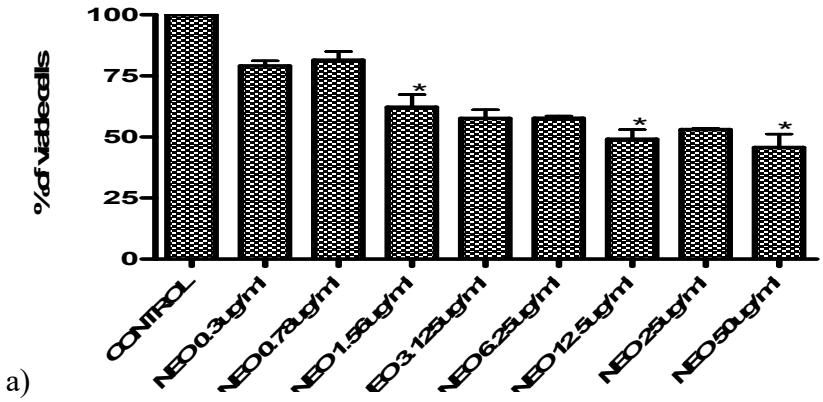
c)

Figure 4.1 a, b and c: The effects of a 72 hour treatment with various concentrations of neodiospyrin, diospyrin and 7-methyljuglone on the survival of THP-1 cells, respectively. The results of three experiments with three replicates in each are expressed as the mean values, ± SD (*p* values < 0.05 are denoted by the asterisks).

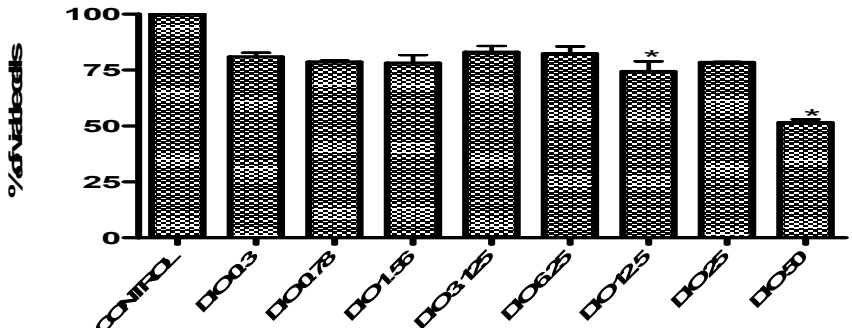
4.6.3 Effects of synthetic 7-methyljuglone, neodiospyrin and diospyrin on the viability of Vero cells

The effects of the test compounds on the viability of African monkey kidney cells are shown in Figure 4.2a, b and c. All 3 compounds caused dose-related inhibition of the growth of the Vero cells, which attained statistical significance at concentrations of

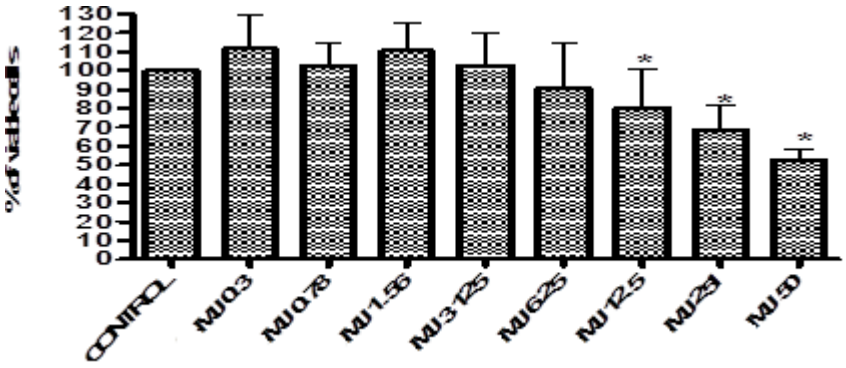
1.56, 12.5 and 12.5µg/ml in the case of neodiospyrin, diospyrin and synthethic 7-methyljuglone respectively.



a)



b)



c) **Figure 4.2 a, b and c: The effects of a 72 hour treatment with various concentrations of neodiospyrin, diospyrin and 7-methyljuglone on the survival of Vero cells, respectively. The results of three experiments with three replicates in**

each are expressed as the mean values, ± SD (*p* value < 0.05 are denoted by the asterisks).

4.7 Discussion

The use of *in vitro* cytotoxicity tests facilitates screening of compounds during early stages in the discovery process (Barlie et al., 1994) and it is estimated that only one in ten potential drug candidates make it through the development process. One-third of these failures is due to unacceptable toxicity levels. Cytotoxicity assays are rapid and cost-effective tools to identify the likely failures before a compound is entered into the costly development process and to help choose the optimal candidate.

However, *in vitro* cytotoxicity assays may not accurately predict *in vivo* toxicity where an integrated physiological system is in place. A newly isolated compound may be highly active against an organism, but could also be very harmful to surrounding tissue that contains the organism. Thus it is important to assess cytotoxicity at different concentrations of the compound to determine the concentration that least affects normal cell proliferation.

At concentrations of up to 6.25µg/ml, none of the test agents significantly affected the growth of THP-1 cells, while at higher concentrations, dose-dependent inhibition of growth was observed with all three compounds. Similar results were observed with Vero cells with the exception of neodiospyrin which caused significant inhibition of growth at 1.56µg/ml. This study was an important pre-requisite to identify the maximum concentrations of the test naphthoquinones which could be used in systems designed to detect the bioactivities of the test naphthoquinones aginst intracellular MTB, using infected THP-1 cells as surrogates for human macrophages. The concentrations chosen

were 6.25µg/ml for all three test compounds, as THP-1 cell viability was unaffected at this concentration, as well as 12.5 and 25µg/ml which possessed moderate cytotoxic activity.

In conclusion, and as mentioned above, the experiments described in this chapter are a necessary forerunner of subsequent experiments designed to establish the efficiency of these agents against MTB resident in infected THP-1 cells. Intracellular bioactivity is a critical property of anti-TB chemotherapeutic agents and preclinical studies of this type represent an important step in the progression of selecting drug candidiates for more intensive pre-clinical evaluation and phase-1 studies. The results presented in the current chapter suggest that the test naphthoquinones should ideally have MIC values for MTB of 6.25µg/ml, preferably lower.

As mentioned, the results presented in this study form the basis for investigating some of the compounds for pre-clinical and clinical studies. Pre-clinical trials and pharmacokinetic studies are required before one can conclude their use for TB-treatment.

5.1 Introduction

Human lungs are subjected to constant environmental exposure to micro-organisms and particles. Since MTB is spread via the aerosol route, the lungs require an appropriate defense mechanism (Leemans et al., 2005). When particulate matter is inhaled, fewer than 10% of particles will reach the respiratory bronchioles and alveoli; most will settle in the upper respiratory epithelium, where they are likely to be expelled by the mucociliary escalator (Nardell et al., 1993). Bacteria that enter the lower respiratory tract are phagocytosed by alveolar macrophages and are either killed or survive to initiate an infection (Dannenberg, 1993).

Because macrophages are the preferred host cell of replication of MTB, the pathogenesis of TB is a complex interaction between the host and pathogen (Leemans et al., 2005). The macrophage plays a key role as an effector cell mediating and activating innate immune responses, as well as initiating the acquired immune response (Widdison, 2007). Few microorganisms can survive inside macrophages, due to the abundance of acidic vacuoles and hydrolytic enzymes. There is evidence that macrophages can destroy *M. tuberculosis* by different mechanisms i.e. delivery of lysosomal enzymes to phagosomes, production of toxic effector molecules, by limiting the availability of iron to mycobacteria, and undergoing apoptosis (Leemans et al., 2005). However, MTB has evolved mechanisms which enable this microbial pathogen to survive and multiply in these cells.

In this chapter the intracellular activities of the 3 compounds, neodiospyrin, diospyrin and synthetic 7-methyljuglone were evaluated against pathogenic (H37Rv) MTB, using the human macrophage cell line (THP-1).

5.2. Materials and methods

Determination of the intracellular bioactivity of the test compounds for *M. tuberculosis* (H37Rv)

5.2.1 Test compounds

Neodiospyrin and diospyrin were provided by Prof N. Lall for the intracellular study. Diospyrin, neodiospyrin and synthesized 7-methyljuglone were dissolved in DMSO to obtain a final concentration of 50mg/ml. Stock solutions were diluted in DMSO and added directly to antibiotic-free RPMI 1640 medium (Sterilab, South Africa) before addition to the microplates, to give final concentrations of 6.25, 12.5 and 25µg/ml. The final concentration of DMSO in the wells was 0.1% for testing against the H37Rv strain of *M. tuberculosis.*

5.2.2. Preparation of *M. tuberculosis* cultures.

5.2.2.1 Materials

The laboratory H37Rv strain of MTB was obtained from the American Type Culture Collection (Rockville, MD) and stock cultures of MTB (H37Rv) prepared at the P3

facilty of the the South African medical Research Council (Pretoria). Sterile inoculation loops, sterile test tubes with glass beads, Middlebrook 7H9 broth (with 5% glycerol), Vortex, McFarland standard #1, disposable cuvettes, Middlebrook 7H10 agar plates supplemented with oleic acid, albumin, dextrose, catalase (OADC) and cryovials were purchased from Microsep, South Africa. This study was conducted at the Medical Reasearch Council TB labs in a P3 containment facility.

5.2.2.2 Slant inoculation

H37Rv cultures were scraped off the stock slant agar with a sterile inoculation loop and spread onto fresh slant agar and incubated for 3 weeks at 37ºC.

5.2.2.3 Preparation of bacterial stock solution:

All the bacteria were scraped from the surface of the slant agars with a sterile inoculation loop, and transferred to the test tube containing glass beads and vortexed for 2 minutes to remove cell clumps. Five ml of Tween 80/saline (See Appendix A) were added to the tube and vortexed for 2 minutes to homogenize the bacteria. The contents were allowed to settle for 15 to 30 minutes before aspirating the supernatant with a pipette and transferring it to an empty test tube. The optical density (OD) of the bacterial suspension was measured using a UV spectrophotometer (Unicam, CA) at a wavelength of 540 nm and diluted to obtain bacterial inocula of 1 x 10^7 cfu/ml. The bacterial inocula were

divided into aliquots of 500µl and stored at -70ºC until use. Colony forming units per ml were determined using the McFarland nephelometer

5.2.3 Culturing of THP-1 cells

5.2.3.1 Reagents

Dulbeccos' Eagle's minimum essential medium, without antibiotics, PMA (phorbol 12-myristate 13-acetate) at 25µg/ml in RPMI-1640 media, 96-well flat-bottommed plate(s), flat-bottom, filter-sterilized Tween-saline (PBS containing 0.05% Tween-80), FCS, 10cc syringes, needles and phosphate-buffered saline (PBS) were used as reagents in this study.

5.2.3.2 Differentiation of THP-1 cells into macrophages:

THP-1 cells were seeded in complete Dulbeccos' Eagle's minimum essential medium at a concentration of $5x10^5$ cells/ml in the wells of a 96-well plate, to which 20µl of PMA was added to the wells to induce the differentiation of monocytes into macrophages. The final volume in each well was 200µl. The plates were incubated for 24hrs at 37ºC, 5% CO_2.

5.2.3.3. Preparation of differentiated cells (macrophages) for infection with *M. tuberculosis*

After incubation, 180µl of supernatant was removed from each well to remove PMA and undifferentiated cells and 200µl of fresh complete media was added to each well and the plates were incubated for 1 hour at 37ºC and 5% CO_2.

5.2.3.4 Infection of differentiated THP-1 cells with *M. tuberculosis*

Macrophage monolayers were infected with MTB at a bacteria:macrophage ratio of 20:1, using 100µl ($9x10^5$ cfu's) of bacterial solution which was added to each well, and the plates incubated at 37ºC in a humidified 5% CO_2 atmosphere, for 4 hours. The wells were washed 3 times with sterile PBS to remove extracellular bacteria, and 180µl media without antibiotic and 20µl compound or medium or vehicle control, in this case 0.1 % DMSO, was added to the relevant wells. Dulbeccos' Eagle's minimum essential medium was added to the wells to give a final concentration of 6.25µg/ml, 12.5µg/ml and 25µg/ml. The plates were then incubated for 5 days at 37ºC in humidified 5% CO_2 atmosphere.

5.2.3.5 Effects on the intracellular survival of MTB in macrophages

After 5 days of incubation, the medium was removed and the intracellular bacteria were released by lysing the macrophages with 0.5 % sodium dodecyl-sulphate (SDS). Survival of bacteria was determined by the radiometric BACTEC method, (Bactec System,Becton Dickinson, USA). BACTEC vials were inoculated with the cell lysates (100µl) and incubated at 37ºC. The change in Growth Index (GI) was recorded daily until the vials containing the 1:100 dilution of the untreated control suspension reached a GI of 30 or more.

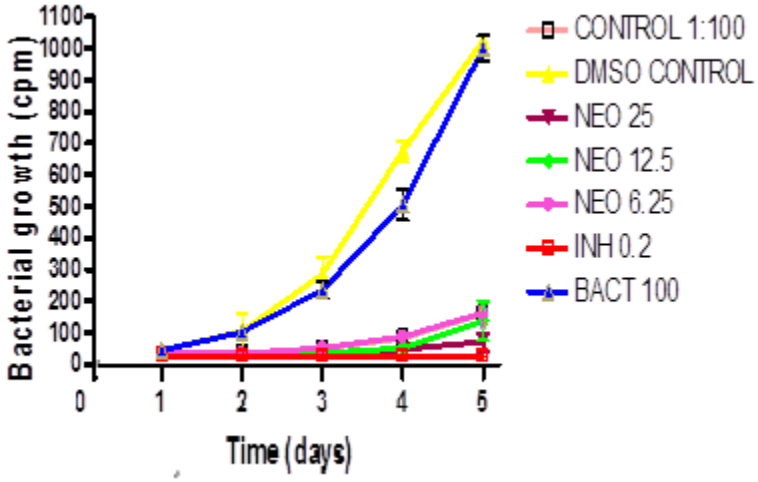
5.3 Statistical analysis

Statistical analysis of the data was conducted as discussed with Dr. P. Becker of the Unit for Biostatistics at the MRC. As mentioned above, experiments were performed at least three times and three replicate vials were used per treatment. The significance of differences between controls and vials treated with compounds were analyzed using the Students paired t-test. Differences with a p-value of < 0.05 were considered to be statistically significant, which was calculated using the combined data for experiments 1 and 2).

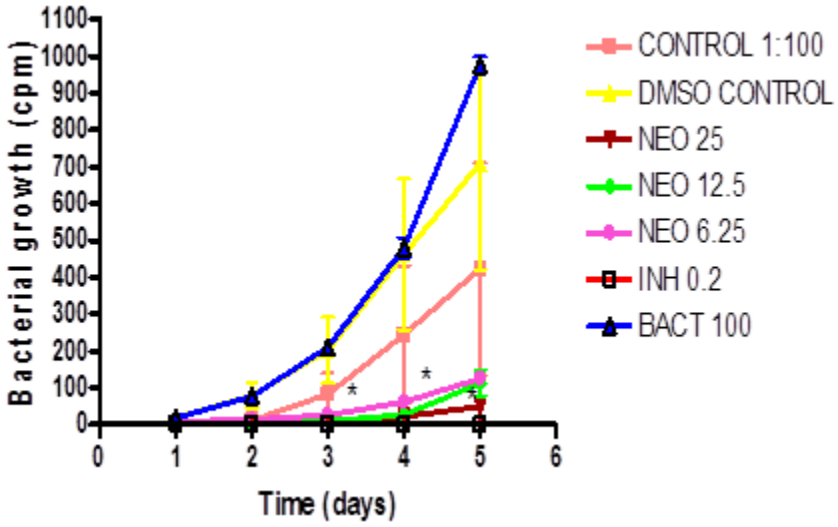
5.4 Results

Once the macrophages were infected with MTB, and transferred to the BACTEC vials, growth was monitored daily until the Δ GI of the 1:100 dilution of bacteria reached 30 or more. INH was used as a control indicated by the blue line in all the Figures. The effects of the compounds intracellularly on the survival of the H37Rv strain of MTB are shown in Figures 5.1a and b, 5.2a and b and 5.3a and b for neodiospyrin, diospyrin and synthetic 7-methyljuglone respectively, in which the data for experiments 1 and 2 correspond to the a and b series respectively. The results clearly show that all 3 compounds i.e. synthetic 7-methyljuglone, diospyrin and neodiospyrin, possess intracellular anti-mycobacterial activity, at all 3 concentrations tested (6.25 µg/ml, 12.5µg/ml and 25µg/ml), which in the case of 6.25ug/ml is not toxic to THP-1 cells. Concentrations of neodiospyrin, diospyrin and synthetic 7-methyljuglone which completely suppressed the intracellular growth of MTB, are shown in Table 5.1, together with their respective, previously reported MIC values using conventional microbiological procedures (Van der Kooy, 2007).

5.4.1.1 Activity of neodiospyrin against intracellular MTB: Experiment 1 and 2



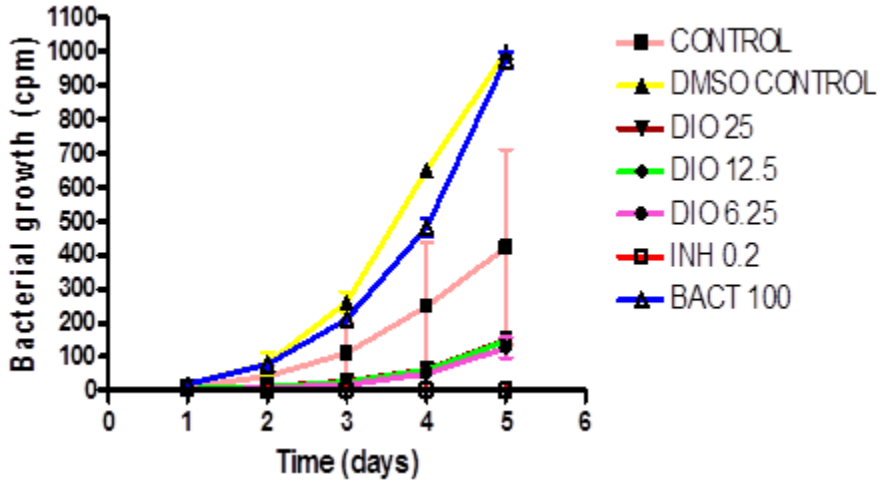
a)



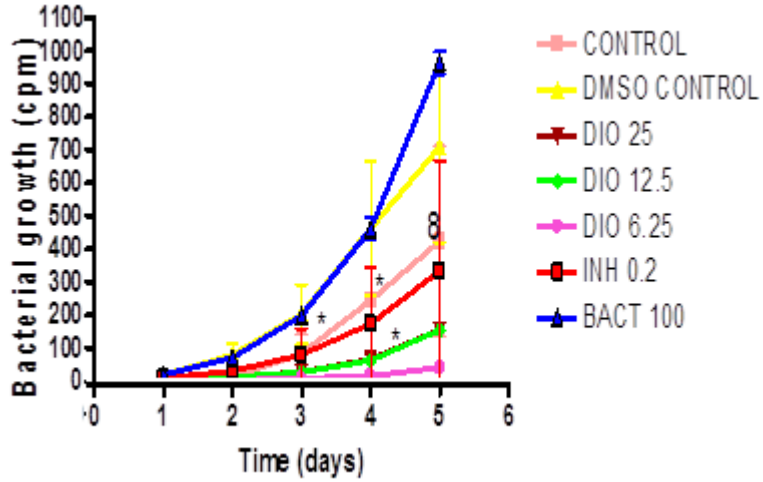
b)

Figure 5.1a and b: Effects of neodiospyrin on intracellular survival of MTB (in radioactive counts per minute). The results of one experiment with three replicates for each system are expressed as the mean values ± SD. *=$p < 0.05$.

5.4.2.1 Activity of diospyrin against intracellular MTB: Experiment 1 and 2



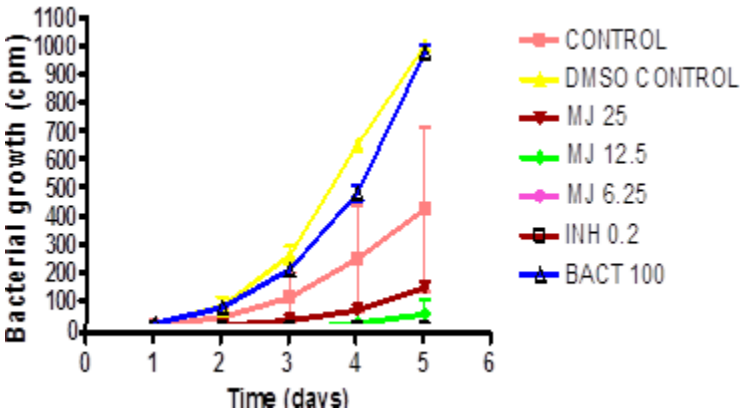
a)



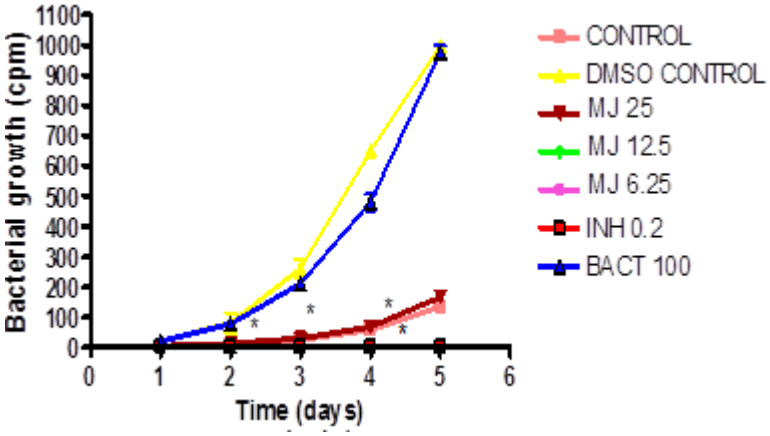
b)

Figure 5.2a and b: Effects of diospyrin on intracellular survival of MTB (in radioactive counts per minute). The results of one experiment with three replicates for each system are expressed as the mean values ± SD. *=$p < 0.05$.

5.4.3.1 Activity of synthetic 7-methyljuglone against intracellular MTB: Experiment 1 and 2



a)



b)

Figure 5.3a and b: Effects of synthetic 7-methyljuglone on intracellular survival of MTB (in radioactive counts per minute). The results of one experiment with three replicates for each system are expressed as the mean values ± SD. *=$p < 0.05$.

Table 5.1 Concentrations of neodiospyrin, diospyrin and synthetic 7-methyljuglone which completely suppressed the intracellular growth of MTB in relation to previously published MIC values.

Compound	Concentration (µg/ml) which totally suppressed intracellular growth of MTB	*Previously published MIC values for MTB ((µg/ml)
Neodiospyrin	12.5	10.0
Diospyrin	25	8.0
Synthetic 7-methyljuglone	6.25	0.5
Control	10	> 10

*** Mahapatra et al., 2007; Van der Kooy et al., 2007**

Table 5.2 Intracellular anti-tuberculosis activity and cytotoxicity of test naphthoquinones

Compound	MIC[a]	IC_{50}[b]	IC_{50}[c]	SI[d]	SI[e]
Neodiospyrin	**12.5**	**>50**	**>50**	**4**	**4**
Diospyrin	**25**	**>50**	**>50**	**2**	**2**
Synthetic 7-methyljuglone	**6.25**	**>50**	**>50**	**8**	**8**
Isolated 7-methyljuglone	**>5**	**>15.1**	**>50***	**30.22**	**100**

[a]-Minimum inhibitory concentration (in this context, this is the concentration of each agent which completely inhibited intracellular growth of MTB)
[b]-Fifty percent inhibitory concentration in Vero cells
[c]- Fifty percent inhibitory concentration in THP-1 cells
[d]-Selectivity index-Vero cells (IC_{50}/MIC)
[e]- Selectivity index-THP-1 cells (IC_{50}/MIC)
*- Bapela et al., 2006

5.5 Discussion

Neodiospyrin and diospyrin, as well as synthetic 7-methyljuglone were evaluated for their intracellular bioactivities against MTB in this study. The purpose of using the 7-methyljuglone which showed good antimicrobial activity in conventional MIC studies done by Lall et al. (1995) is that Chakrabarty et al. (2001), showed that analogs of a compound sometimes have a higher activity than the compound itself. Also, it takes a long time and huge quantities of root material from *Euclea natalensis* to isolate pure 7-methyljuglone, and since the method of synthesising 7-methyljuglone is much faster and

yields higher quantities of synthetic 7-methyljuglone, synthetic 7-methyljuglone was evaluated in the current study.

The concentration of synthetic 7-methyljuglone (0.5µg/ml) which completely suppressed the intracellular growth of MTB was very similar to that of isolated 7-methyljuglone which was between 2.5µg/ml and 5µg/ml for the H37Rv strain of MTB as reported by Bapela et al. in 2005. If the MIC of synthetic 7-methyljuglone is compared to the conventional anti-TB agent, pyrazinamide, a first line anti-tuberculosis agent, for which 40µg/ml was shown to be the lowest active dose using human macrophages as reported by Sbarbaro et al. (1996), synthetic 7-methyljuglone has much more potent activity intracellularly than pyrazinamide.

Diospyrin showed similar intracellular activity (MIC 25 µg/ml) to that reported by van der Kooy and colleagues, (MIC of > 10µg/ml) (van der Kooy, 2006). Neodiospyrin was found to be more active intracellularly than diospyrin. In the case of neodiospyrin, the concentration which completely suppressed growth was 12.5µg/ml.

The results of this study demonstrated that neodiospyrin, diospyrin and synthetic 7-methyljuglone are active against intracellular MTB, which may underpin the use of root bark of *Euclea natalensis* for treatment of pulmonary infections as claimed by traditional healers and the Zulu and Shangaan people of South Africa. Also, isolated 7-methyljuglone showed a higher SI value than synthetic 7-methyljuglone and therefore appears to be more active of the two compounds. However, in the therapeutic setting, the

synthethic compound which can be produced in greater quantities is likely to be the preferred compound.

However, apparent limitations of these agents, which are evident from the studies described in the previous chapter and this chapter are:

i) the concentrations of the 3 test agents required to completely inhibit the growth of intracellular MTB in THP-1 cells are close to (synthetic 7-methyljuglone), equivalent to (neodiospyrin) or greater (diospyrin) than those at with cytotoxic effects on this macrophage cell line were observed; and

ii) the concentrations at which complete inhibition of growth was detected in the THP-1 system are equivalent to (neodiospyrin) or higher (diospyrin, synthetic 7-methyljuglone) than the conventional MIC values, suggesting that cytotoxicity or poor cellular uptake/intracellular bioavailability may restrict the intracellular activity of these agents. Ideally, an anti-mycobaterial agent should: i) have a high level of intracellular accumulation; ii) be bioactive intracellularly; and iii) act in unison with the intrinsic antimicrobial systems of the infected macrophage. Alternatively, binding of the naphthoquinones to proteins in the added fetal calf serum may restrict the interaction of these agents with the MTB-infected macrophages.

The studies described in the following chapters were designed to identify the cellular site and the mechanism of anti-mycobacterial action of the test agents, neodiospyrin, diospyrin and synthetic 7-methyljuglone in MTB and *M. smegmatis.*

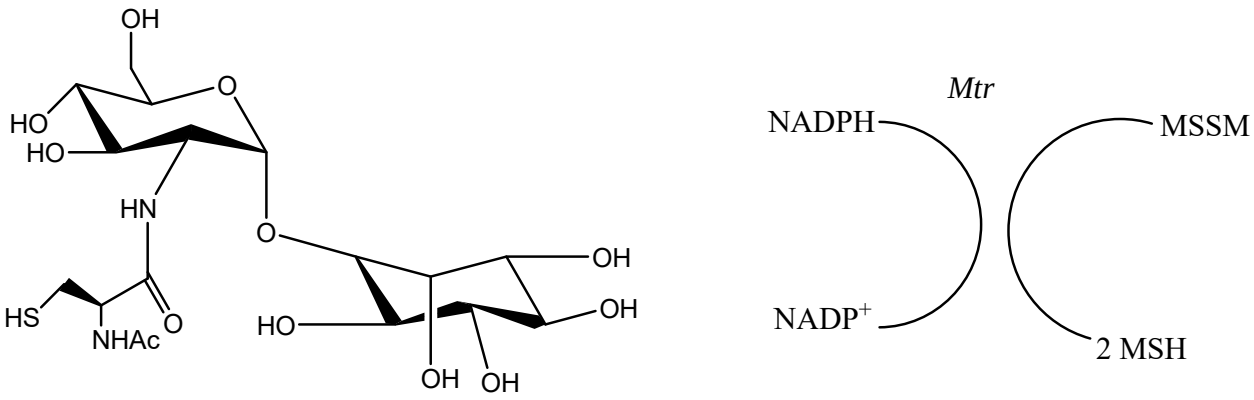
Apart from providing mechanistic insights, information generated from these studies may assist in the design of strategies to improve the antimicrobial activities of the test naphthoquinones.

6.1 Introduction

The results presented in the previous chapter confirm and extend the findings of Lall et al., (2001) and van der Kooy et al., (2007) that *Euclea natalensis*-derived naphthoquinones possess anti-mycobacterial properties. However, the mode of antibacterial action of naphthoquinones remains to be conclusively established. Several mechanisms have been proposed to explain the antimicrobial and anti-viral activities of naphthoquinones such as plumbagin, menadione and juglone these include: i) antibacterial acitivity, possibly due to the high lipid solubility of the naphthoquinones, enabling them to act as carriers of certain sulfonamides by facilitating the diffusion of these compounds through the cell wall, giving them a better opportunity to exert their effects on MTB (Roushidi et al., 1976; Osman et al., 1983); ii) antiviral activity, as naphthoquinones act as non-peptidic inhibitors of HIV-1 protease, an enzyme essential for replication of HIV, by binding to the active-site groove of HIV-1 protease (Brinkworth et al., 1995); iii) trypanocidal acivity, by acting as efficient and subversive substrates of *Trypanosoma cruzi* lipoamide dehydrogenase by binding at the dimmer interface of *Trypanosoma cruzi* (Salmon et al., 2001) and iv) anticancer activity, by inhibition of *in vivo* growth growth of Erlich Ascites Carcinoma through inhibition of respiration and disruption of the whole cell resulting in expulsion of intracellular material leading to total cell lysis (Hazra et al., 1984). More recently, Lall et al., (2007) investigated the effects of 7-methyljuglone, as well as a large series of derivatives of this naphthoquinone, on MTB mycothiol disulphide reductase in relation to their anti-mycobacterial activities (Mahapatra et al., 2007). A number of the test compounds were found to function as subversive substrates with mycothiol disulphide reductase. MTB

lacks glutathione; instead it maintains millimolar concentrations of the structurally distinct low molecular weight thiol, mycothiol (MSH) (Spies et al., 1994; Newton et al., 1995; Fahey, 2001). Glutathione, a major endogenous antioxidant, synthesized by most eukaryotes including humans, plays a fundamental role in the management of oxidative stress (Copley, 2002, Grill, 2001). MTB and other actinomycetes do not synthesize gluthathione, but instead produce the thiol, mycothiol (MSH) which parallels the function of glutathione. MSH is required for growth of MTB (Newton, 1995) by assisting in maintaining a reducing intracellular environment. MSH is oxidized to the symmetrical disulfide (MSSM). Mycothiol reductase (Mtr), an NADPH-dependent enzyme reduces MSSM back to MSH, reducing the oxidative stress within the intracellular environment (Patel et al., 1999, Sareen et al., 2003). A study conducted by Newton et al., (1995) and Rawat et al., (2002) showed that mycobacteria that lacked MSH exhibited increased sensitivity to oxidative stress, making this redox pathway a potential biological target for development of future antitubercular drugs. Furthermore, the mycothiol disulphide redox pathway is unique to MTB. However, subversive substrate efficiency with mycothiol disulfide reductase was generally noted at relatively high concentrations of these substituted naphthoquinones with no clear correlations with their anti-mycobacterial activities (Mahapatra et al., 2007). The mycothiol/disulphide reductase pathway and the futile redox cycle of naphthoquinones are shown in Figure 6.1.

a)



Mycothiol (MSH)

b)

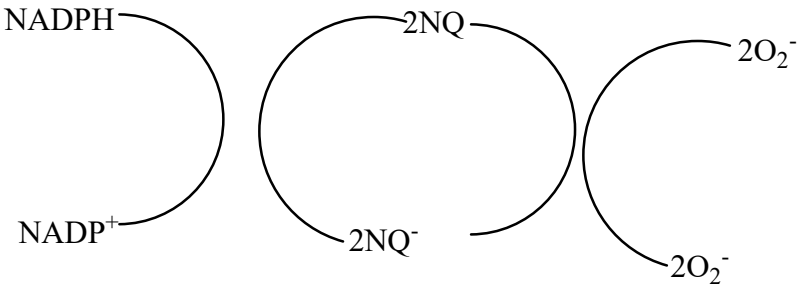


Figure 6.1 a) Mycothiol and its disulfide reductase; b) futile enzymatic redox cycle of naphthoquinones (Mahapatra, 2007)

The studies described in the current chapter were designed with the primary objective of investigating the effects of the test naphthoquinones on two relatively unexplored potential targets: a) the functional integrity of the mycobacterial membrane; and b) microbial energy metabolism. Membrane integrity and function were determined according to the uptake and exclusion of potassium (K^+) and calcium (Ca^{2+}) respectively,

while energy metabolism was assessed by measurement of microbial ATP (a*denosine triphosphate)* levels.

6.2 Materials and methods

6.2.1 Antimicrobial agents

Test compounds were provided by Prof N. Lall. Neodiospyrin, diospyrin and synthetic 7-methyljuglone were dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 1mg/ml. Subsequent dilutions were made in DMSO and neodiospyrin, diospyrin and synthetic 7-methyljuglone were used at final concentrations ranging from 0.045µg/ml to 25µg/ml. Appropriate solvent controls were included in the various assays described.

6.2.2 Chemicals and reagents

Unless otherwise indicated, all chemicals used were obtained from the Sigma Chemical Co. (St Louis, MO, USA). Radiochemicals were purchased from Perkin Elmer Life and Analytical Sciences, (Shelton, CT, USA).

6.2.3 Bacteria

Two bacterial strains, *M. tuberculosis* H37Rv (MTB), and *M. smegmatis were* used, both of which were provided by the Tuberculosis Research Institute of the South African Medical Research Council (Pretoria, South Africa). MTB was grown for three days at

37ºC in an OADC (oleic acid, albumin dextrose, catalase)-enriched Middlebrook 7H9 nutrient broth (Difco, Detroit, MI, USA) supplemented with 0.05% Tween 20, while *M. smegmatis* was cultured overnight in similar conditions. The final albumin concentration of the culture medium was 5mg/ml.

6.3 Potassium (K^+) transport studies

$^{86}Rb^+$ (rubidium-86 chloride, 37 MBq, Perkin Elmer Radiochemicals) was used as a tracer for measuring K^+ uptake. $^{86}Rb^+$ was used instead of $^{42}K^+$ which is a strong gamma-emmitter, but has a short half-life of only 12.4 hours. $^{42}K^+$ has been described in several previous studies as being a useful tracer for the measurement of microbial transport of K^+ (de Bruyn et al., 1996; Parish and Stoker, 2000, Blondelet-Rouault et al., 1997; Hinds et al,1999). In the present study, $^{86}Rb^+$ was used as a tracer to probe mycobacterial K^+ transport. In this study rubidium was used instead of potassium as potassium and rubidium are similar in their chemistry and ionic radii (hydrated ions approximately 2 A.; anhydrous ions= 1.34 and 1.49 A., respectively), $^{86}Rb^+$ has been used as a tracer for potassium in soil/plant studies (Deist and Talibudeen, 1966)

MTB and *M. smegmatis* bacterial (MC 2115) strains were cultured for 3 days and overnight respectively in Middlebrook 7H9 medium supplemented with OADC as mentioned previously, before being harvested, pelleted by centrifugation (2500xg for 15min), washed and resuspended in glucose and K^+-free buffer to deplete intracellular K^+ (KONO buffer KONO buffer (46 mM Na_2HPO_4, 23 mM NaH_2PO_2, 0.4 mM $MgSO_4$, 0.6 mM $FeSO_4$ and H_2O) , pH 7.4, modified from Epstein and Kim, 1971) and adjusted

turbidometrically to a concentration of 1 x 10^8 cfu/ml. The mycobacteria were treated with neodiospyrin, diospyrin or synthetic 7-methyljuglone at concentrations ranging from 0.045µg/ml to 25µg/ml for 30 minutes at 37ºC in this minimal media, followed by the addition of $^{86}Rb^+$ (2µCi/ml) and glucose (22mM) and further incubation for 45min for M. smegmatis and 90min for MTB. Appropriate solvent controls were included. The final volume in each tube was 10ml and the final concentration of bacteria was 1 x 10^7 cfu/ml for *M. smegmatis* and 2 x 10^7 cfu/ml for MTB. After incubation, the bacteria were washed twice with ice-cold phosphate-buffered saline (PBS, 0.15M, pH 7.4, containing 100 mM cold K^+ to stop the reaction) and bacterial pellets disrupted by adding 0.4ml warm 5% trichloroacetic acid (TCA). Radioactivity was assayed in a liquid scintillation spectrometer (Tri-Carb 2100TR, Packard Instrument Company, Meridien, CT, USA) and, net uptake of K^+, expressed as radioactive counts per minute (cpm), was taken as the difference in uptake of $^{86}Rb^+$ in the tubes incubated at 37ºC and the controls kept on ice.

Additional experiments were performed to determine the possible influence of added protein (bovine serum albumin, BSA at a final concentration of 5mg/ml) on naphthoquinone–mediated inhibition of the uptake of K^+ by MTB and M. smegmatis. The experimental design was identical to that described above, the only exception being the inclusion of BSA, which was added to the bacteria prior to exposure to the test naphthoquinones.

6.4. Calcium (Ca^{2+}) uptake studies

These were performed using *M. smegmatis* only. The bacteria were cultured overnight as described in the preceding section, pelletted by centrifugation and resuspended in Ca^{2+} -free Hanks balanced salt solution (HBSS, Highveld Biological (Pty) Ltd, Johannesburg, South Africa) at a concentration of 1 x 10^7 cfu/ml. The bacteria were then pre-incubated for 5min at 37ºC in Ca^{2+}-free HBSS containing 5 µCi/ml of radiolabelled calcium (Calcium-45 chloride, 863.58 MBq/mg, Perkin Elmer Radiochemicals), followed by addition of the test naphthoquinones at a fixed, final concentration of 1.5µg/ml, or an equivalent volume of DMSO solvent to control systems. The final volume in each 15ml conical tube was 3ml, and the tubes were then incubated for a further 60min at 37ºC. Following incubation, 11ml of ice-cold Ca^{2+}replete ($CaCl_2$) HBSS was added to each tube, and the tubes then placed in an ice-bath to stop the reactions. The bacteria were then pelletted by centrifugation, washed once with 15ml of ice cold Ca^{2+}-replete HBSS, and the bacterial pellets disrupted by addition of 0.4ml warm 5% TCA and radioactivity determined using the liquid scintillation spectrometer as described above. Because of the minimal differences between the background system held on ice and systems incubated at 37ºC, these results are not expressed as the differential values, but rather as the absolute counts per minutes (cpm).

6.5 Adenosine triphosphate (ATP) levels

Microbial ATP concentrations were determined using a sensitive luciferin–luciferase chemiluminescence method (de Bruyn et al., 1996; Gordhan et al., 1996). After injection of firefly lantern extract into solutions containing ATP, light is emitted for several

minutes. Maximum light intensity is reached after two seconds. The light intensity of the initial flash is found to be directly proportional to the amount of ATP present up to a final concentration of 2µM. In the reaction of firefly bioluminescence, luciferin is converted to luciferyl adenylate by ATP in the presence of magnesium, and thereafter luciferyl adenylate is oxidized by molecular oxygen resulting in the emission of a yellow-green light. These reactions are catalyzed by luciferase.

The effects of neodiospyrin, diospyrin and synthetic 7-methyljuglone on the ATP levels of MTB and *M. smegmatis* were assessed using the Bac Titre Glo™ microbial cell viability assay (Promega, Madison, WI, USA), which is based on the principles described above.

M. smegmatis and MTB were cultured as mentioned in 6.2.3. The bacterial cells 1 x 10^7cfu/ml in 10 ml KONO buffer were co-incubated for 60 minutes at 37 ºC with or without the test compounds (0.023µg/ml -12.5µg/ml). The cells were concentrated by centrifugation, the pellets were then mixed with an equal volume of the Bac Titre Glo ™ solution which contains the bacteriolytic constituent, and then incubated for 5min at room temperature and assayed for ATP over a 10 second period, using a $20/20^n$ chemiluminometer (Turner Biosystems Inc, Sunnyvale, CA, USA). The results are expressed as relative light units.

6.6. Determination of the minimum inhibitory concentration (MIC) of the test naphthoquinones for *M. smegmatis*

M. smegmatis was cultured as mentioned in 6.2.3. The mycobacteria were pelleted by centrifugation (2500 g/15min) and resuspended in the growth media (Middlebrook 7H9 nutrient broth enriched with OADC) to a numerically standardised optical density. *M. smegmatis*, at a final concentration of 5 x 10^5 cells/ml, were exposed to the different naphthoquinones (1µg/ml-100µg/ml) for a period of 16h at 37°C in a shaking incubator (200 rpm) (Daihan Labtech, Kyongi-Do, Korea). Following the incubation period, the MIC's were determined by plating the exposed mycobacteria on Middlebrook 7H10 nutrient agar plates enriched with OADC, which were then incubated in a humidified, 5% CO_2, 37°C incubator until growth could visually be detected. The MIC was taken as the lowest naphthoquinone concentration at which growth was completely inhibited. A series of 3 different experiments with 3 replicates in each system were performed. Due to possible protein binding of the compounds, this method was modified as described below.

Using the modified method, the mycobacteria were pelleted by centrifugation (2500g/15min) at room temperature and thereafter adjusted turbidometrically and resuspended in Middlebrook 7H9 nutrient broth (Beckton Dickinson, Cockeyville, MD, USA) without added OADC i.e. essentially protein-free to obtain a final concentration of 2-3 x 10^6 cells/ml. Varying concentrations (ranging from 1µg/ml to 100µg/ml) of test compound or the solvent control system were then added to the bacteria in 15 ml conical

tubes which were incubated in a shaking incubator (200rpm) at 37ºC for 24h, before viability was determined using nutrient agar plates. These plates were incubated in a humidified, 5% CO_2, 37ºC incubator, until growth could be visually detected. The MIC was defined as the lowest concentration of the test compound that completely inhibited the growth of the mycobacteria.

6.7. Protein determination

To test for possible antagonism of the anti-mycobacterial activity of the test naphthoquinones by binding of these agents to proteins present in the bacteriologal culture media, the protein content of these (Middlebrook 7H9 medium without and with added OADC) were measured using the BCA™ procedure. The BCA™ Protein assay kit is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein and was used to determine the total protein contents of the bacterial culture media. A microplate method was used in which 25µl of OADC-enriched or unsupplemented Middlebrook 7H9 media undiluted or serial dilutions of up to 1:32 were pipetted into the wells of microplates. Two hundred microlitres (200µl) of the working reagent was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37ºC for 30 minutes. Thereafter, the plate was cooled to room temperature and the absorbance measured at 562nm on a microplate reader. A standard curve was prepared by plotting the average blank corrected 562nm measurement for each BSA standard vs. its

concentration in µg/ml. The standard curve was used to determine the protein concentration of the OADC-enriched and unsupplemented Middlebrook 7H9 medium.

6.8 Statistical analysis

The results are expressed as the mean ± standard deviation (SD) for between 3 and 5 experiments, with at least 3 replicates for each concentration of the test agents or control systems in each experiment. Levels of statistical significance were calculated using the Student's paired t-test. Differences were considered significant if the probability value was less than 0.05.

6.9 Results

6.9.1 Effects of the test compounds on K^+ transport in the H37Rv strain of *M. tuberculosis* and *M. smegmatis*

The naphthoquinones used in this study were found to cause significant inhibition of the uptake of $^{86}Rb^+$ at all the concentrations studied. The effects of neodiospyrin (0.045-25µg/ml), diospyrin (0.023-1.5µg/ml) and synthetic 7-methyljuglone (0.023-1.5µg/ml) on the influx of $^{86}Rb^+$ are shown in Figures 6.1, 6.2 and 6.3, and Figures 6.4, 6.5 and 6.6 for MTB and *M. smegmatis* respectively. All 3 compounds caused dose-related inhibition of the net uptake of $^{86}Rb^+$ by both MTB and *M. smegmatis* which was maximal at concentrations of 0.375-1.5µg/ml in the case of diospyrin and synthetic 7-methyljuglone,

while neodiospyrin, although extremely effective, did not achieve 100% inhibition even at the highest concentration tested.

The effects of neodiospyrin, diospyrin and synthetic 7-methyljuglone on the uptake of $^{86}Rb^{+}$ by *M. tuberculosis* H37Rv and *M. smegmatis* suspended in KONO with and without BSA are shown in Figures 6.7 and 6.8. In the presence of BSA, naphthoquinone-mediated, inhibition of K^{+} transport was significantly less than in the corresponding BSA-free systems, indicating that protein interferes with the interaction of the naphthoquinones with the mycobacteria.

6.9.2 Effects of the test compounds on uptake of calcium (Ca^{2+}) influx by *M. smegmatis*

Treatment of *M. smegmatis* with the test compounds, at a fixed concentration of 1.5µg/ml, had no effect on the influx of $^{45}Ca^{2+}$ into the mycobacteria as shown in Table 6.1.

Table 6.1: Effects of test compounds on the uptake of $^{45}Ca^{2+}$ by *M. smegmatis*

Systems	***Uptake of $^{45}Ca^{2+}$ by M. smegmatis ****	***Uptake of $^{45}Ca^{2+}$ by M. smegmatis ****
	Experiment 1	**Experiment 2**
Ice Control	2875 ± 284.2	
Control 37 ºC	2673 ± 361.5	4347 ± 389.1
Neodiospyrin	2807 ± 497.9	4393 ± 297.3
Diospyrin	3051 ± 178.9	4239 ± 320.52
Synthetic 7-methyljuglone	2832 ± 429.9	ND

* Results expressed as radioactive cpm (mean ± SD) for 2 different experiments with 5 replicates for each system in each experiment.

ND=not done

6.9.3 Effects of the test compounds on ATP levels in the H37Rv strain of *M. tuberculosis* and *M. smegmatis*

The effects of neodiospyrin (0.045-12.5µg/ml), diospyrin (0.023-1.5µg/ml) and synthetic 7-methyljuglone (0.023-1.5µg/ml) on bacterial ATP concentrations are shown in Figure 6.9, 6.10, 6.11 and Figures 6.12, 6.13, 6.14 for MTB and *M. smegmatis* respectively. In the case of MTB, all three test compounds, at the lower end of the concentration range, caused significant increases in microbial ATP levels, while significant decreases were observed at higher concentrations tested (threshold values of 12.5, 1.5 and 0.375µg/ml

for neodiospyrin, diospyrin and synthetic 7-methyljuglone respectively). Treatment of *M. smegmatis* with neodiospyrin and diospyrin resulted in dose-related increases in ATP levels with no detectable inhibition, while synthetic 7-methyljuglone caused a significant decrease in ATP at the highest concentration tested and an increase at lower concentrations.

6.9.4 Determination of the minimum inhibitory concentration (MIC) of the test compounds for *M. smegmatis*

Using the conventional MIC procedure, none of the test compounds, at the concentration of up to 100µg/ml, affected the growth of *M. smegmatis*. Using the modified procedure without added OADC, however, there was definite inhibition of bacterial growth. The MIC's were 7µg/ml, 6µg/ml, and 6µg/ml for neodiospyrin, diospyrin and synthetic 7-methyljuglone, respectively as shown in Table 6.2. The corresponding MIC values for MTB as previously described by others (Lall et al., 2005; van der Kooy, 2005) are also shown in Table 6.2.

6.9.5 Protein determination

The protein contents of Middlebrook 7H9 nutrient broth with and without OADC were 5290µg/ml and 22µg/ml respectively.

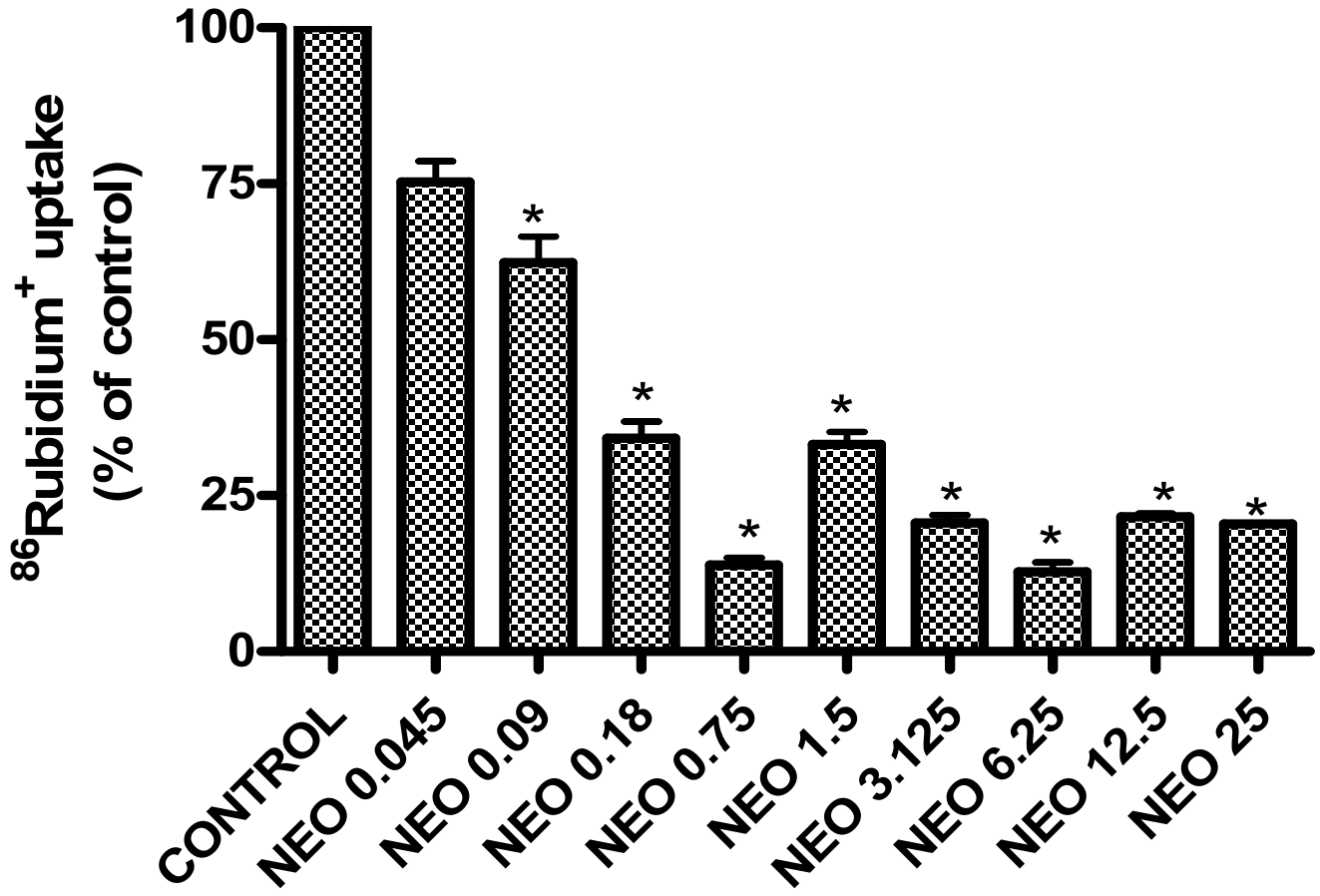


Figure 6.2: The effect of neodiospyrin (NEO) on the uptake of K^+ by the H37Rv strain of MTB. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 106930 counts per minute. * = *p* values < 0.05 when compared to the solvent control system.

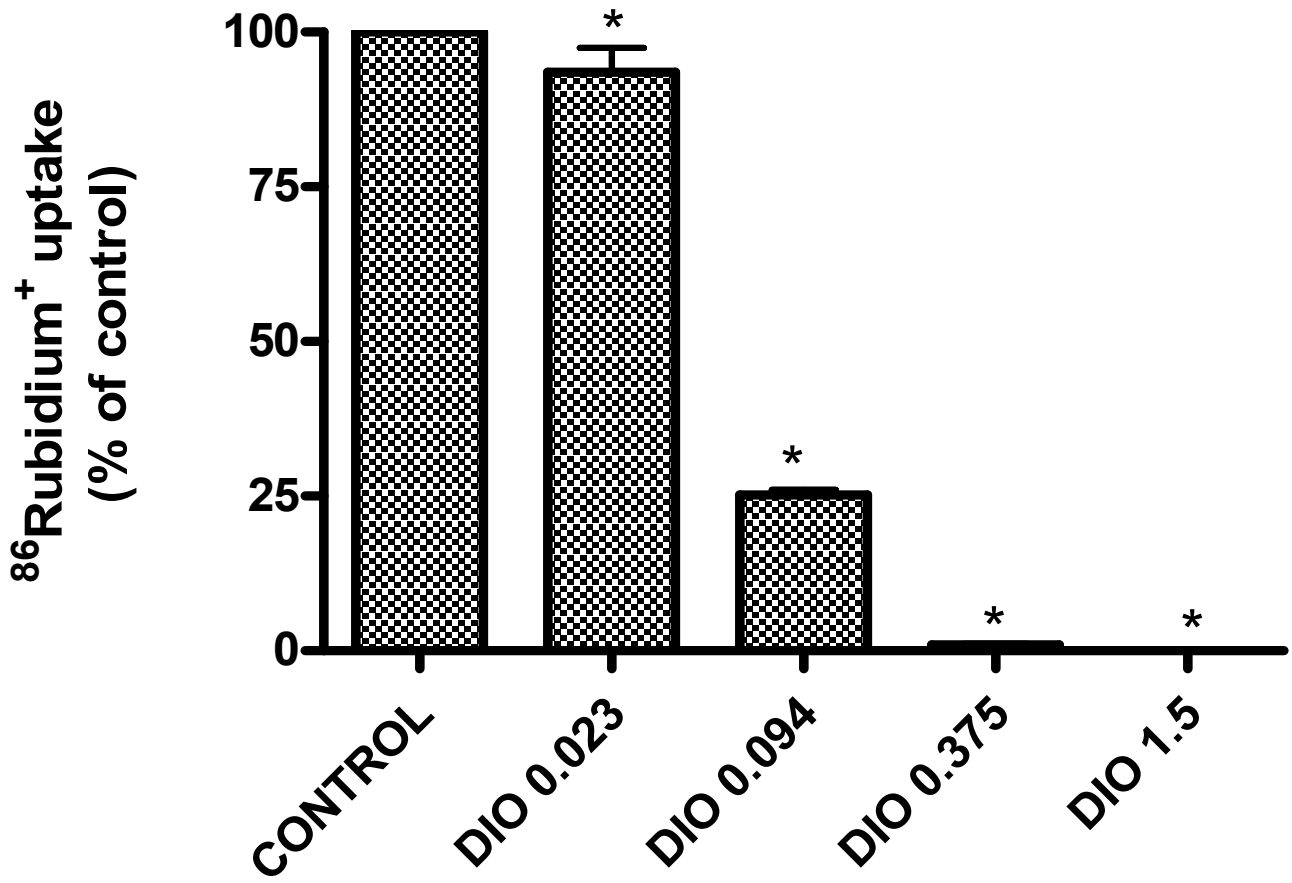


Figure 6.3: The effect of diospyrin (DIO) on the uptake of K^+ by the H37Rv strain of MTB. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 100859 counts per minute. * = *p* values < 0.05 when compared to the solvent control system.

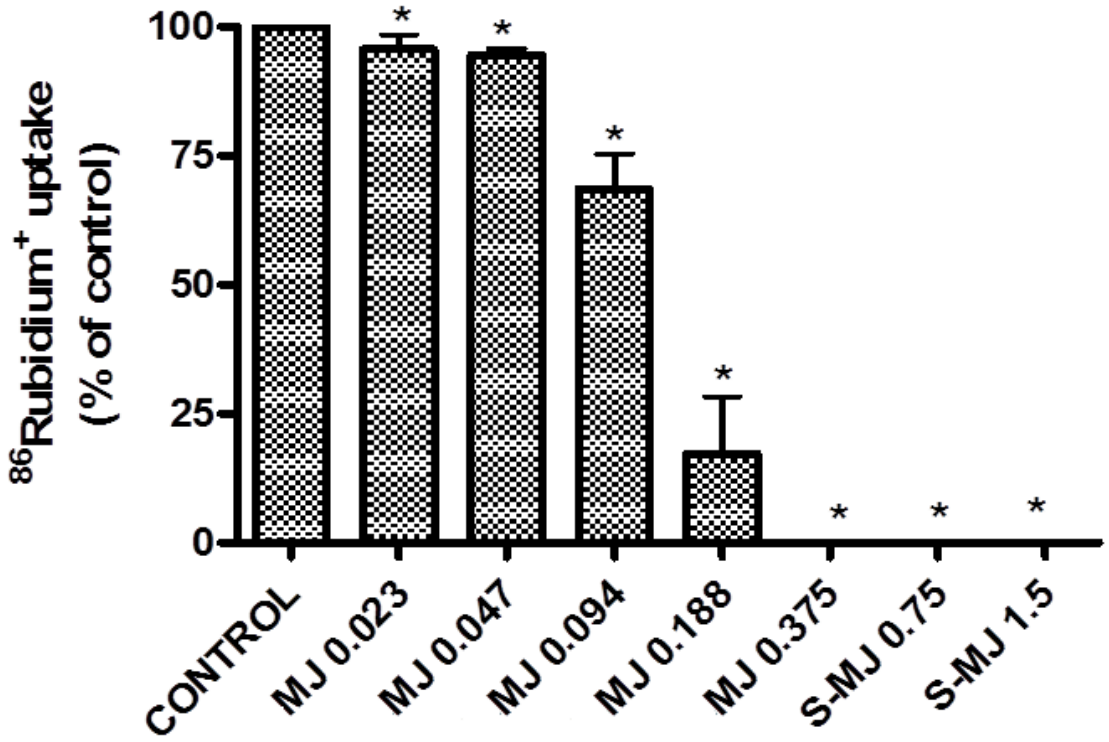


Figure 6.4: The effect of synthetic 7-methyljuglone (S-MJ) on the uptake of K^+ by the H37Rv strain of MTB. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 100859 counts per minute. * = *p* values < 0.05 when compared to the solvent control system.

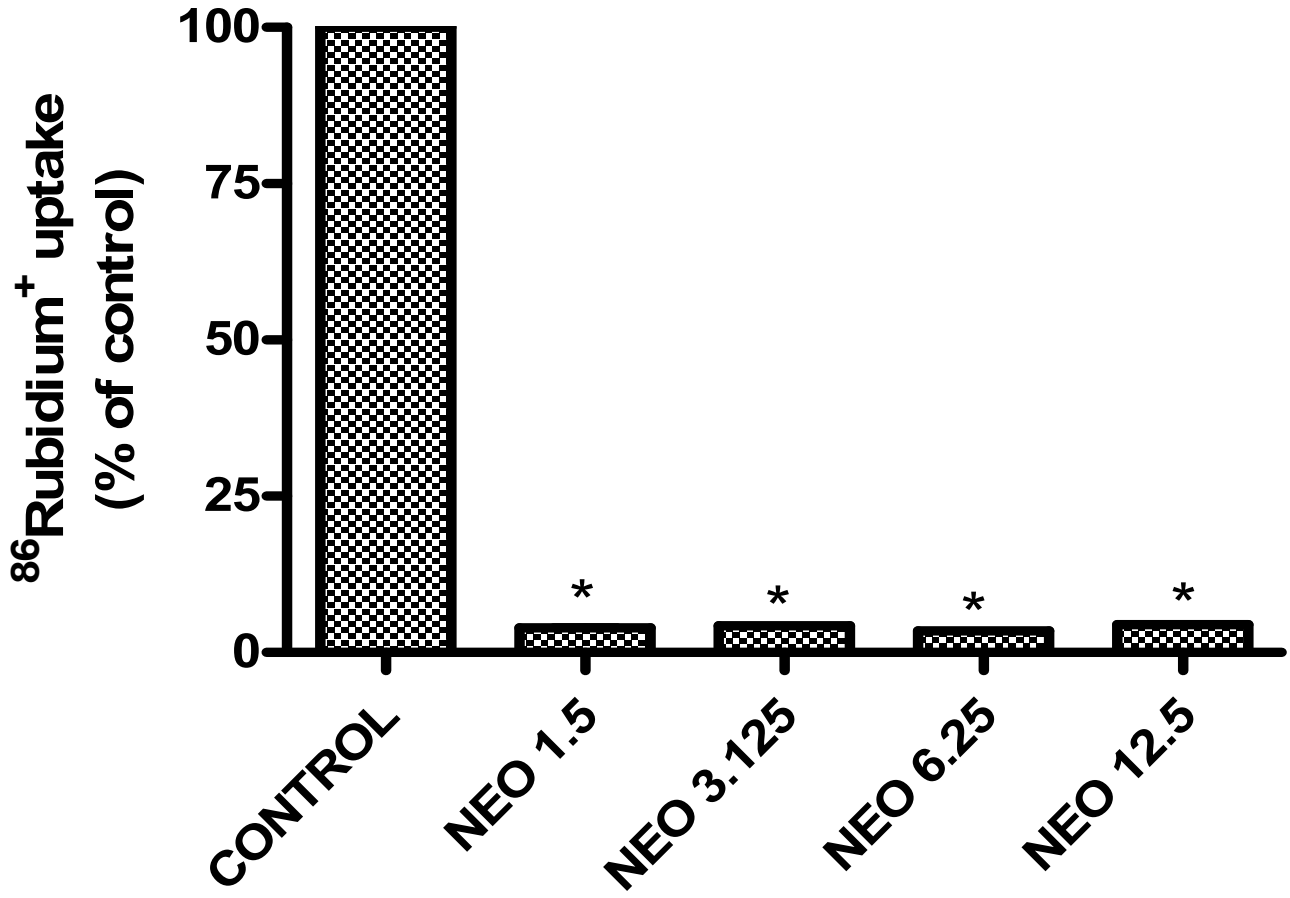


Figure 6.5: The effect of neodiospyrin (NEO), on the uptake of K^+ by *M. smegmatis*. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 83128 counts per minute. * = *p* values < 0.05 when compared to the solvent control system.

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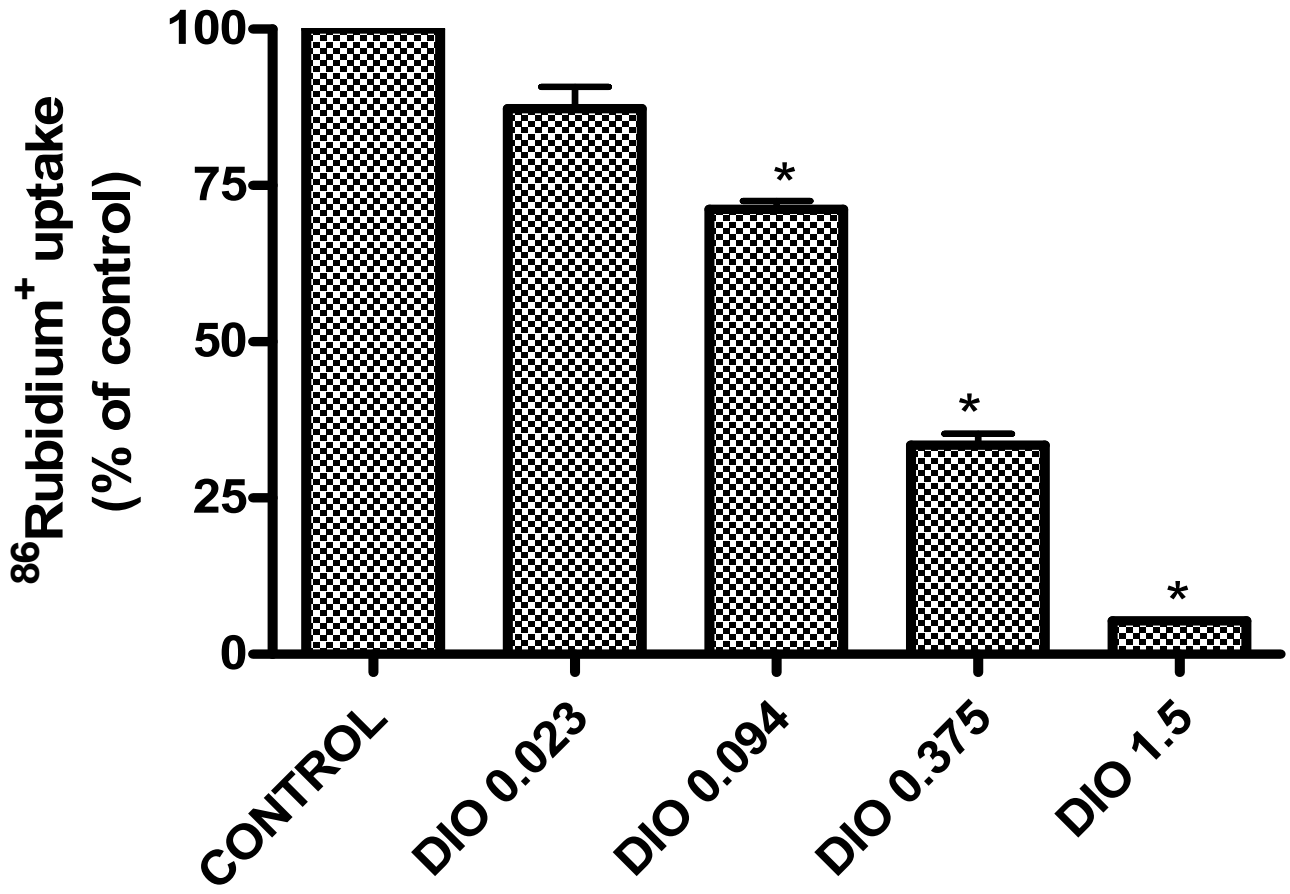

Figure 6.6: The effect of diospyrin (DIO), on the uptake of K^+ by *M. smegmatis*. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 98595 counts per minute. * = *p* values < 0.05 when compared to the solvent control system.

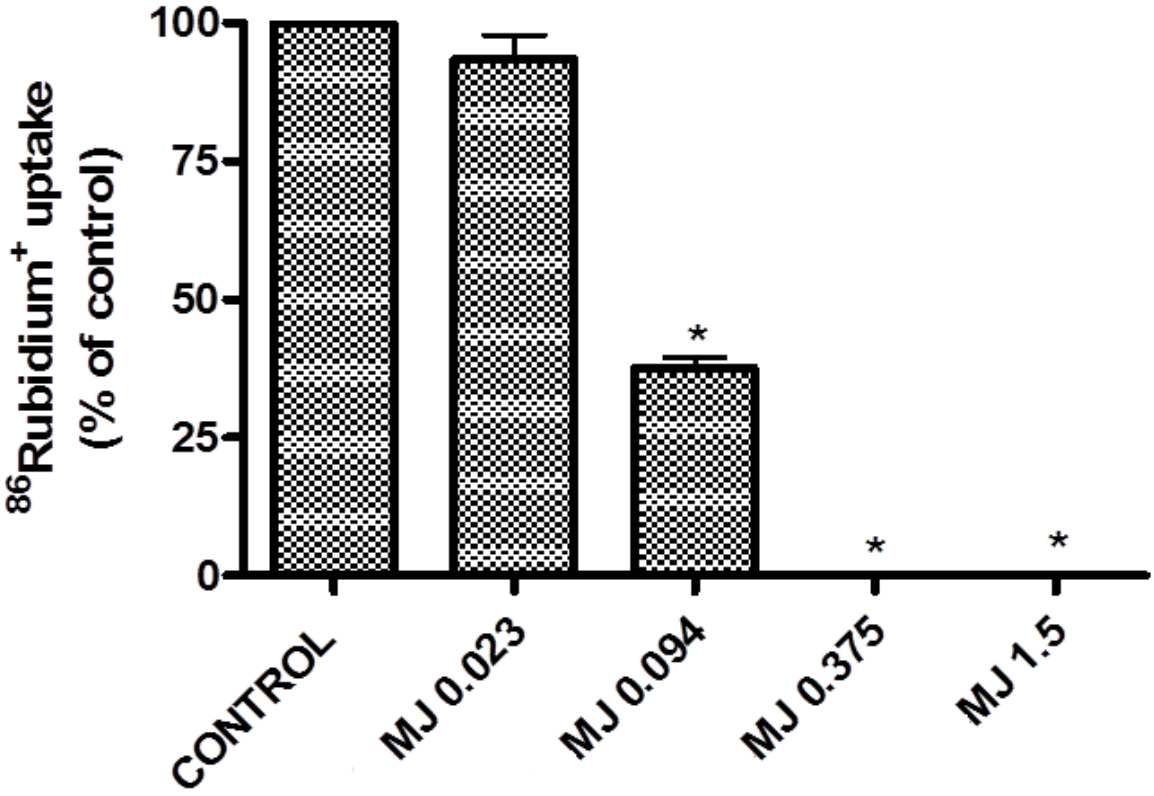

Figure 6.7: The effect of synthetic 7-methyljuglone (S-MJ), on the uptake of K^+ by *M. smegmatis*. The results are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends in each. The results are expressed as the mean percentages for uptake of $^{86}Rb^+$ of the corresponding compound-free control systems ± SD; the absolute value of the control system is 98595 counts per minute. * = *p* values < 0.05 when compared to the solvent control system.

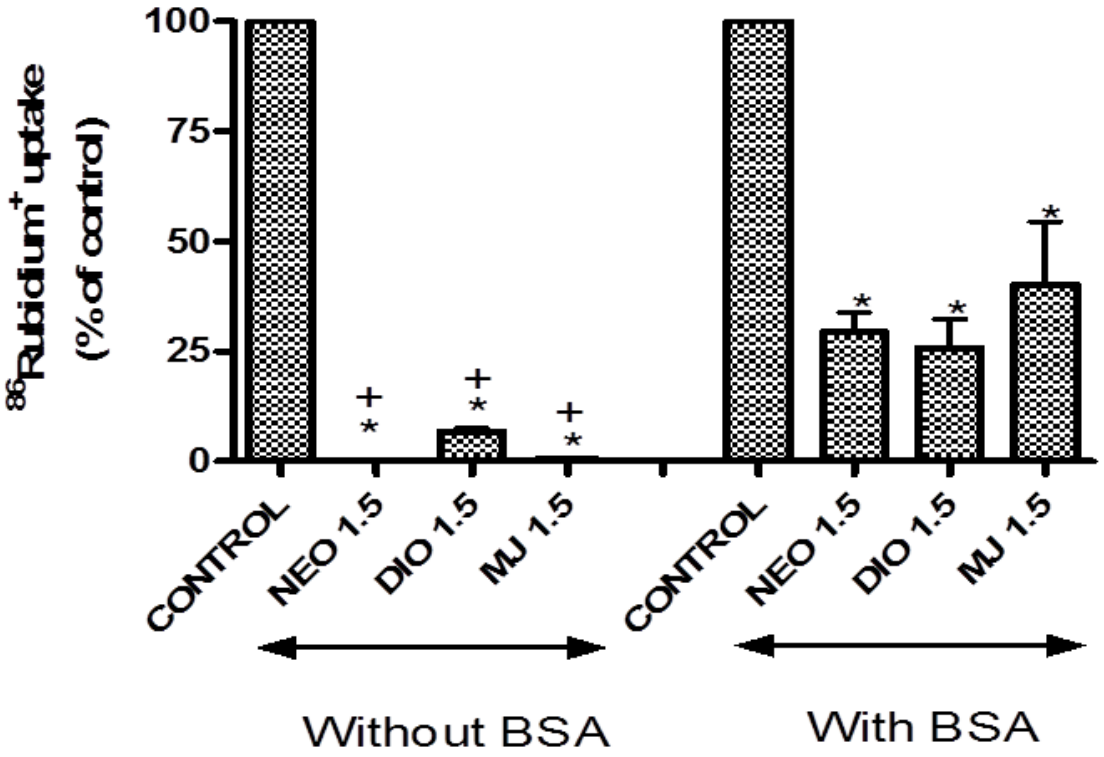

Figure 6.8: The effect of neodiospyrin (NEO), diospyrin (DIO) and synthetic 7-methyljuglone (S-MJ) on the uptake of K^+ by the H37Rv strain of *M .tuberculosis* in KONO with and without BSA. The data shown are from one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound free control systems ± SD; the absolute values of control systems with and without BSA are 58995 and 86317 cpm respectively. * =*p* values < 0.05 when compared to the solvent control; += *p* values < 0.05 for comparison of the sytems without and with BSA.

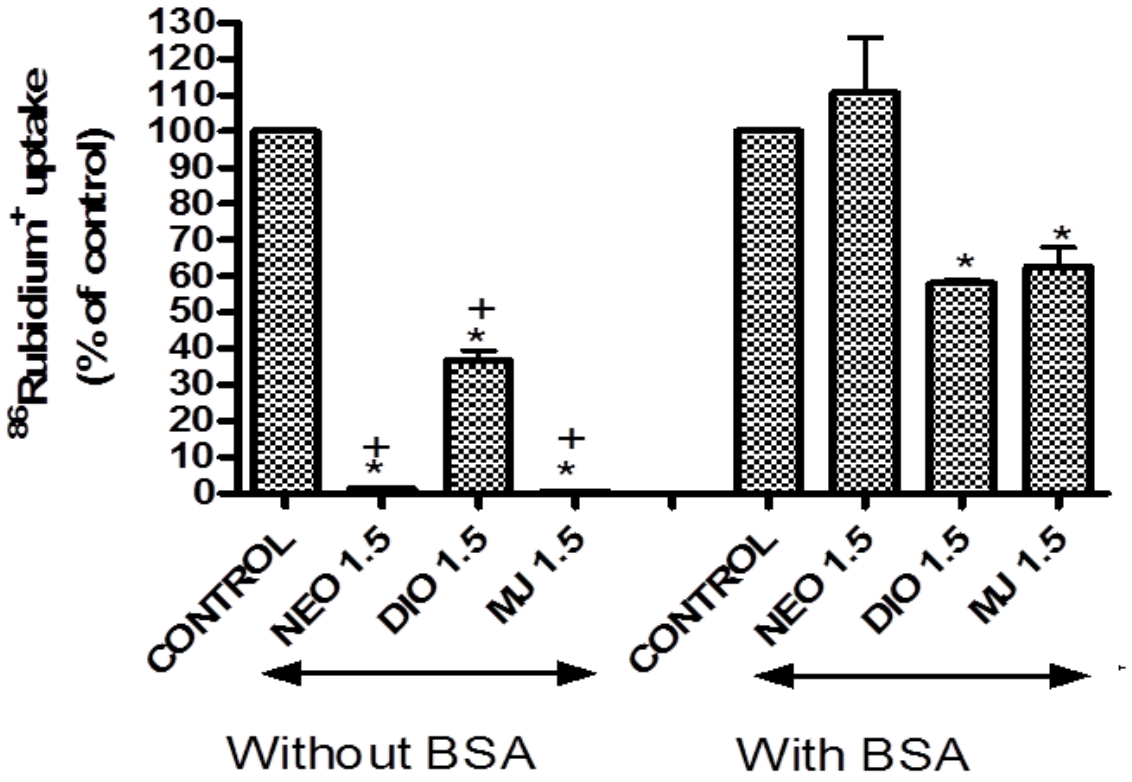

Figure 6.9: The effect of neodiospyrin (NEO), diospyrin (DIO), synthetic 7-methyljuglone (S-MJ), and on the uptake of K^+ by *M. smegmatis* with and without BSA. The data shown are from one experiment with five replicates and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound free control systems ± SD; the absolute values of control systems with and without BSA are 58995 and 86317 cpm respectively. * =*p* values < 0.05 when compared to the solvent control; += *p* values < 0.05 for comparison of the sytems without and with BSA.

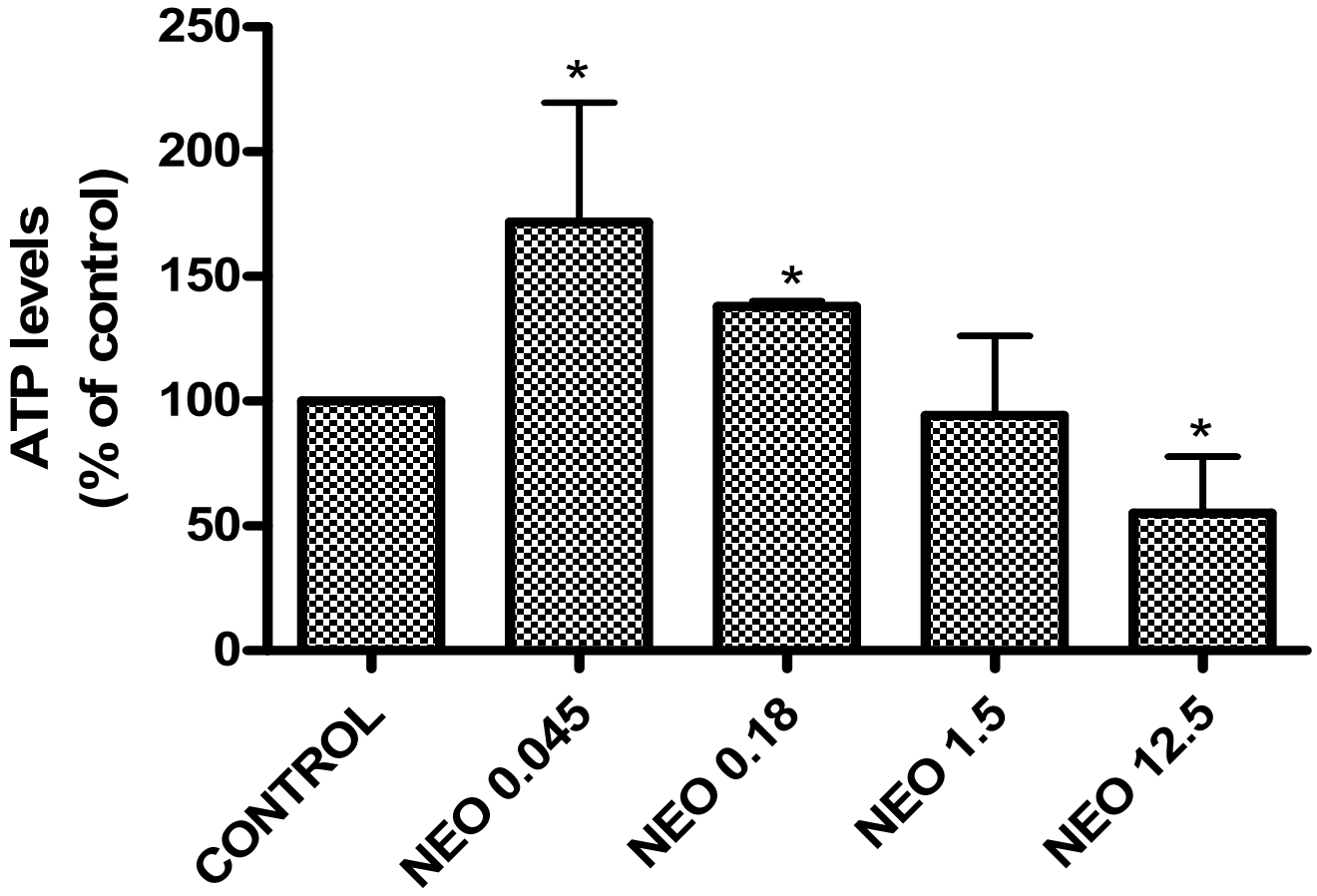

Figure 6.10: The effect of neodiospyrin (NEO) on the levels of ATP in the H37Rv strain of MTB. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ± SD; the absolute value of control is 2306410 relative light units. *= *p* values < 0.05 when compared to the solvent control.

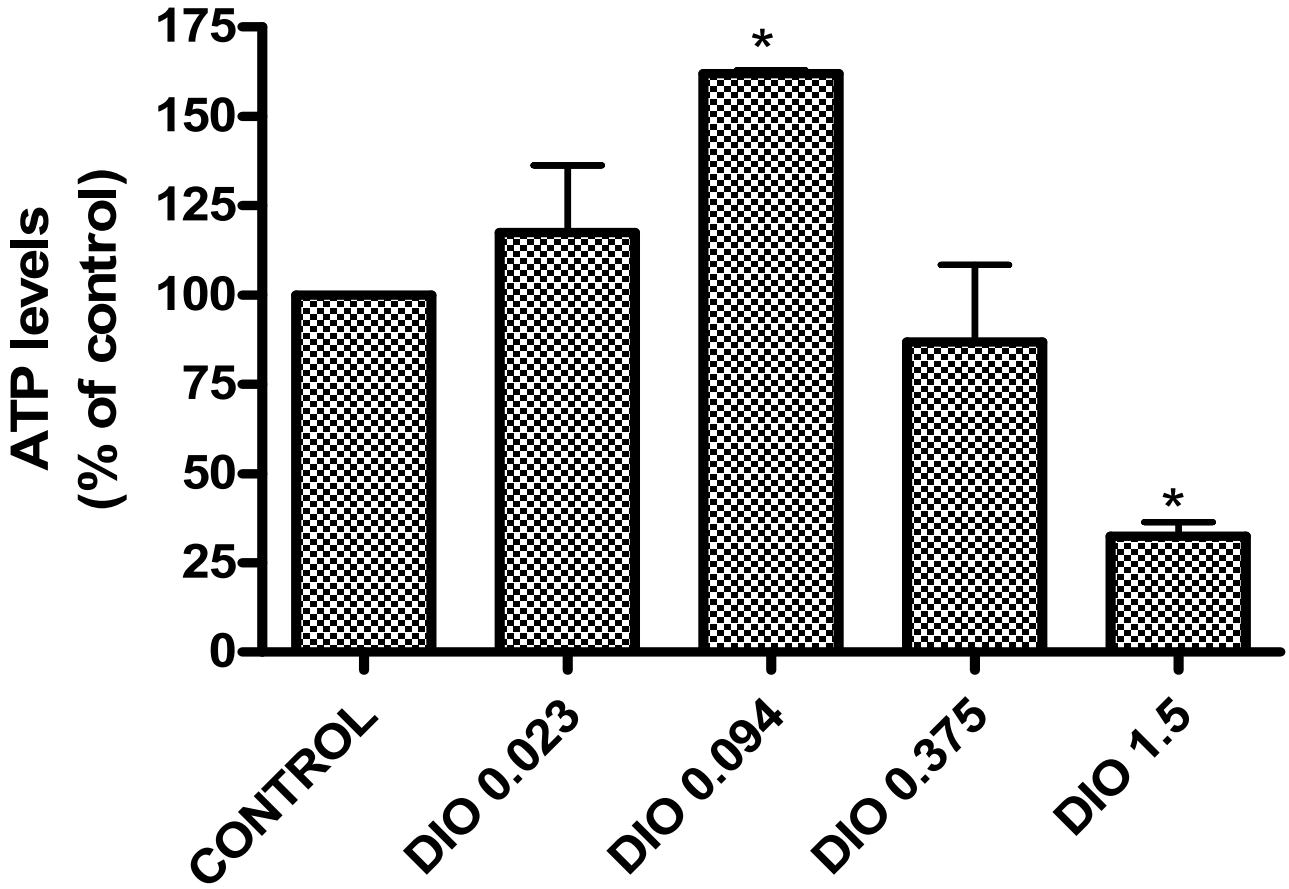

Figure 6.11: The effect of diospyrin (DIO) on the levels of ATP in the H37Rv strain of MTB. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ± SD; the absolute value of control is 2306410 relative light units. *= *p* values < 0.05 when compared to the solvent control.

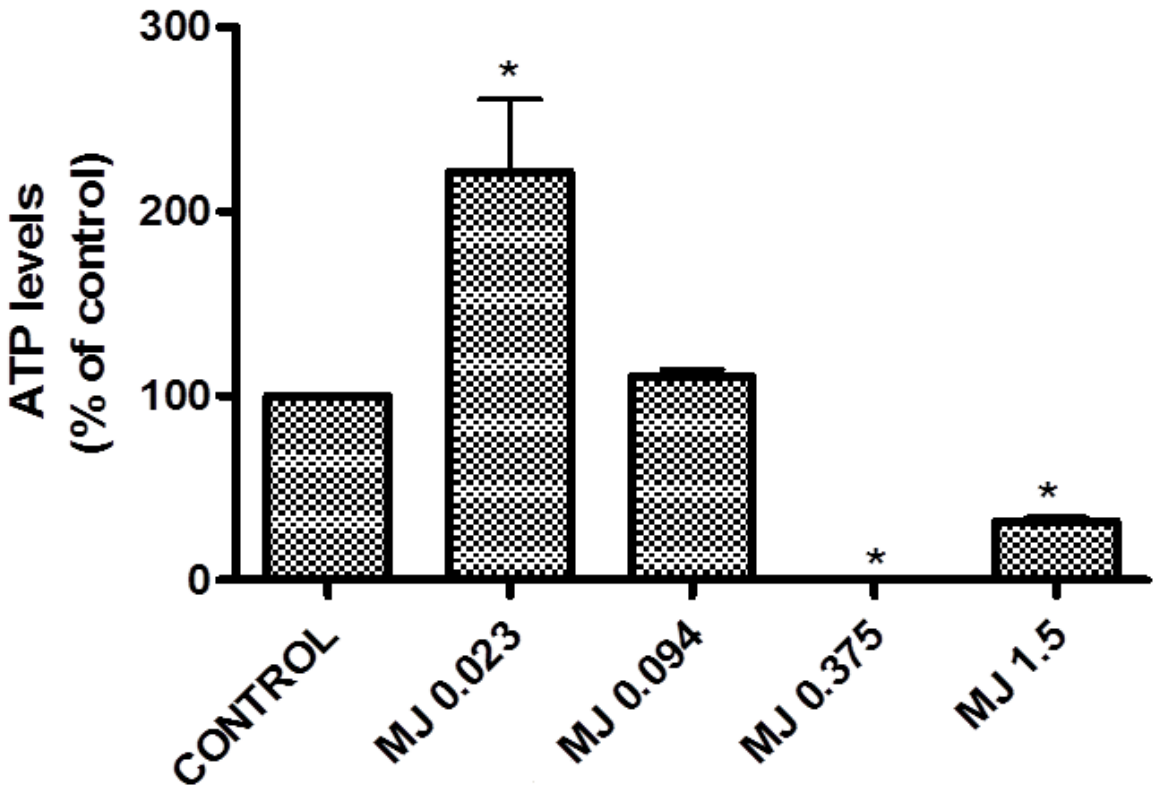

Figure 6.12: The effect of synthetic 7-methyljuglone (S-MJ) on the levels of ATP in the H37Rv strain of MTB. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ± SD; the absolute value of control is 2306410 relative light units. *= *p* values < 0.05 when compared to the solvent control.

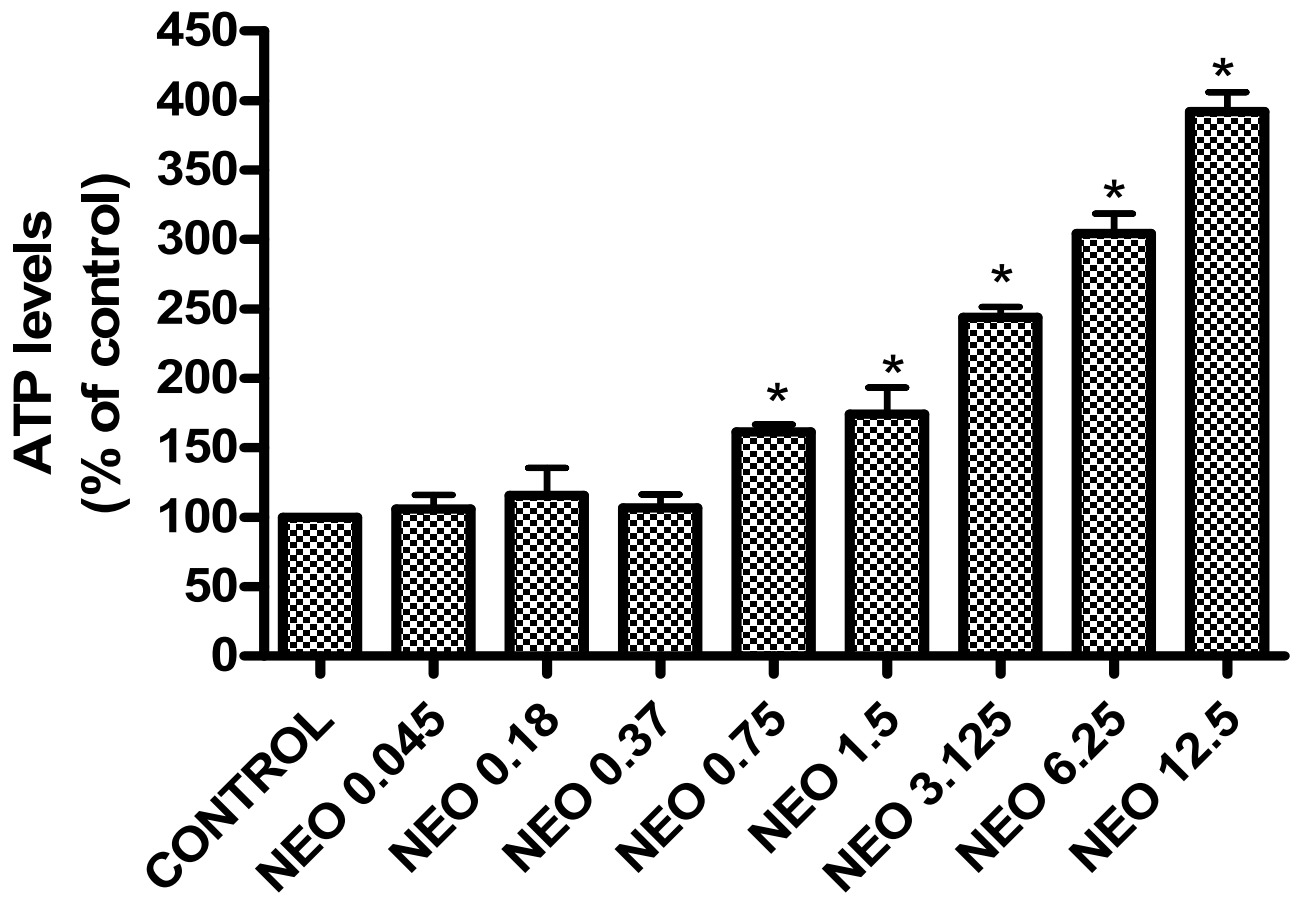

Figure 6.13: The effect of neodiospyrin (NEO) on the levels of ATP in *M. smegmatis*. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ± SD; the absolute value of control is 4894379 relative light units. *= *p* values < 0.05 when compared to the solvent control.

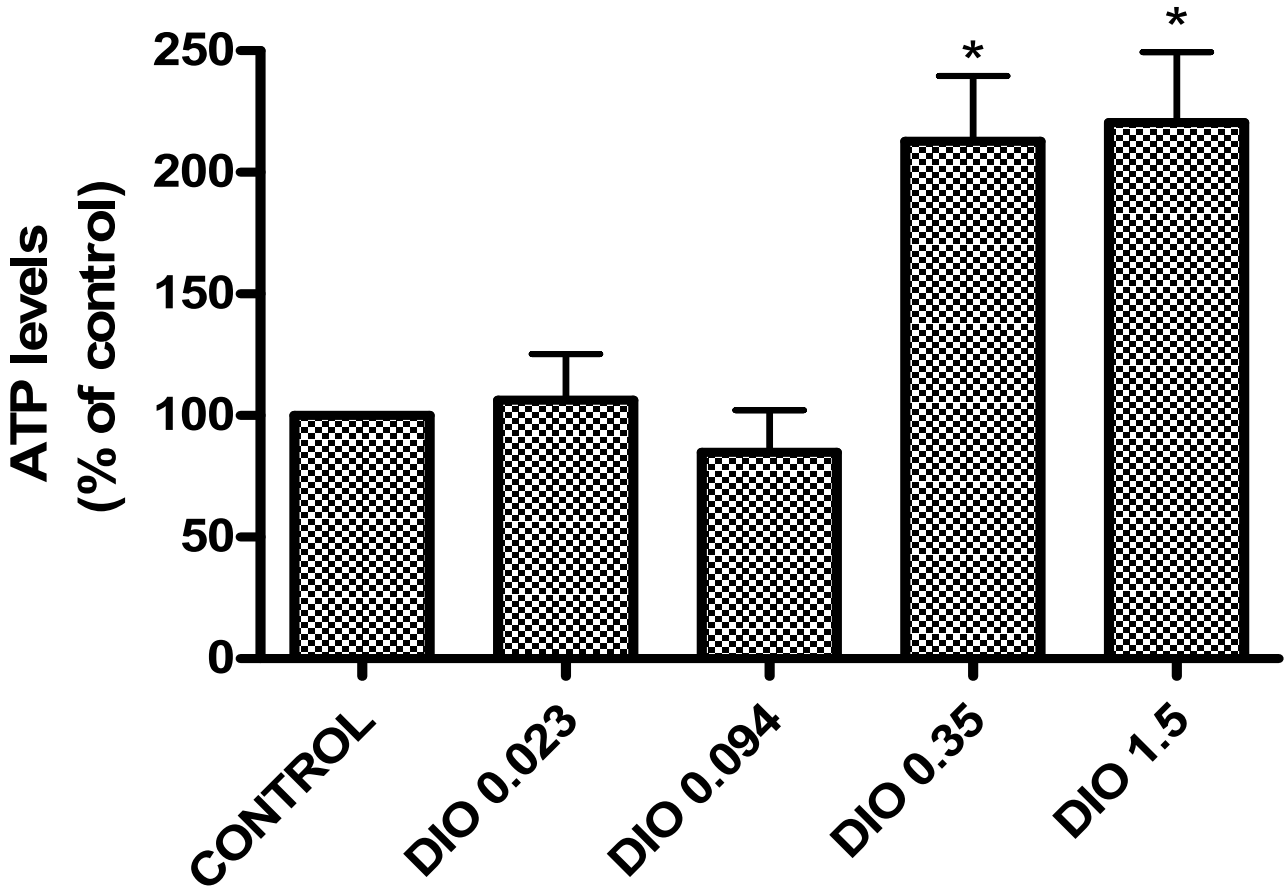

Figure 6.14: The effect of diospyrin (DIO) on the levels of ATP in *M. smegmatis*. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ± SD; the absolute value of control is 4894379 relative light units. *= *p* values < 0.05 when compared to the solvent control.

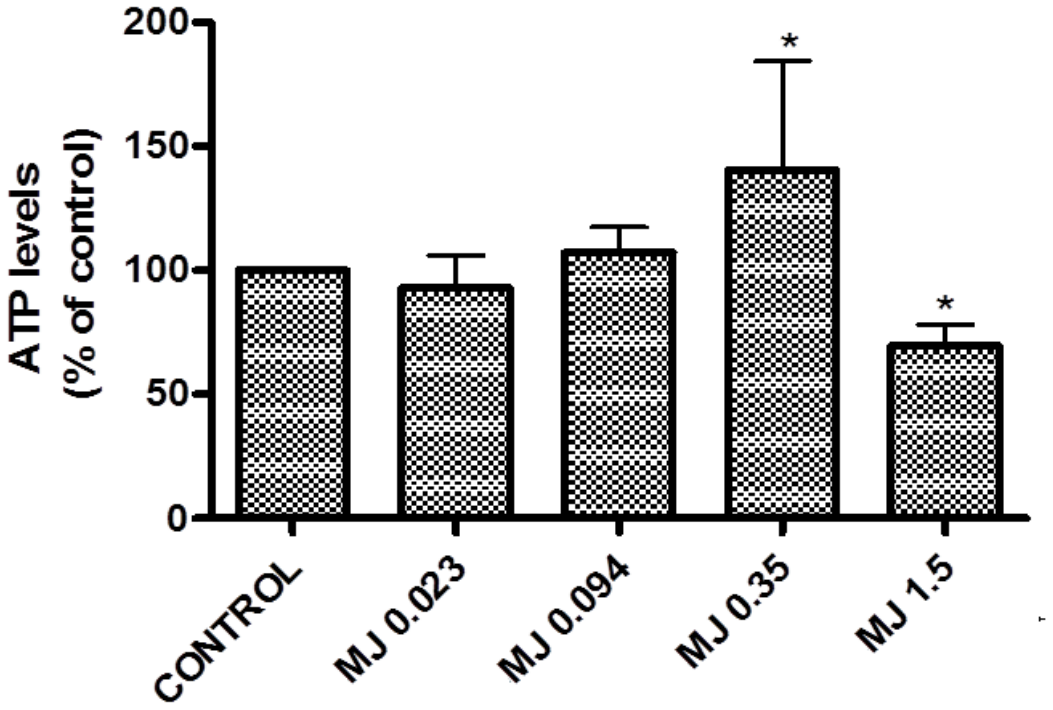

Figure 6.15: The effect of synthetic 7-methyljuglone (S-MJ) on the levels of ATP in *M. smegmatis*. The data shown are that of one experiment with five replicates for each concentration and are representative of 3 different experiments showing similar trends. The results are expressed as the mean percentages of the corresponding compound-free control systems ± SD; the absolute value of control is 4894379 relative light units. *= *p* values < 0.05 when compared to the solvent control.

Table 6.2 MICs of the test compounds for MTB and *M. smegmatis*

Compound	*MIC for MTB (ug/ml)**	*MIC for M. smegmatis (ug/ml)+*
Neodiospyrin	8	7
Diospyrin	10	6
Synthetic 7-methyljuglone	0.5	6

*-These are the values reported by Lall et al., (2005)

+ Results using the modified MIC procedure

6.10 Discussion

To date, very little is known about the mechanism of anti-mycobacterial action of naphthoquinones. The effects of these compounds on K^+ transport and ATP levels in MTB and *M. smegmatis* have not yet been described in previous studies and are the focus of the research described in the current chapter.

Potassium is a major cellular cation that plays a key role in maintaining the cell turgor pressure, cytoplasmic pH, and the activity and expression of enzymes. Active K^+ transporters are vital for bacterial growth and survival in the host and the natural environment. Bacteria often have multiple K^+ uptake systems, and two of those systems (Kdp and Trk) have been characterized in MTB (Bossemeyer et al, 1989). These K^+ transporters of MTB represent potential targets for antimicrobial therapy.

In this study, all three compounds caused potent dose–related inhibition of K^+ uptake by MTB and *M. smegmatis,* which was almost complete at the concentration of 1.5µg/ml. However, inhibition of K^+ uptake occurred at concentrations of all 3 test agents which are lower than their corresponding MIC and intracellular bioactivity values. This discrepancy may be explained by the relatively brief exposure of the bacteria to the test compounds in the K^+ transport assay system in a cell suspending medium (KONO) containing no added protein. In the relatively complex bacteriological growth media, binding of the naphthoquinones to proteins and/or interference with the interaction of these agents with the bacteria by other components of the culture media may reduce their antimicrobial efficacy. In addition, auto-oxidation of the naphthoquinones with the resultant loss of

antimicrobial activity may contribute to the reduced efficacy of these agents in the assays of bacterial growth, which are of considerably longer duration than the assay of K^+ uptake. In support of this contention, van der Kooy (2007) showed that the naphthoquinone, 7-methyljuglone was inactivated, presumably by auto-oxidation after 30 minutes in DMSO solvent and only 75% of the compound remained intact, while in the case of diospyrin, only 68.9% remained intact after 1 day (van der Kooy, 2007).

Two strategies were used to test the potential of protein, or possibly other components of the bacteriological culture media, to antagonize the anti-mycobacterial actions of the test naphthoquinones. Firstly, to measure the effects of these agents on uptake of K^+ by MTB and *M. smegmatis* suspended in KONO without and with added protein; and secondly to expose *M. smegmatis* to the test naphthoquinones in Middlebrook 7H9 nutient broth without and with added OADC containing 5mg/ml albumin.

Inclusion of protein in the KONO medium caused partial, but nevertheless statistically significant, attenuation of the inhibitory effects of all 3 test naphthoquinones (at a fixed concentration of 1.5µg/ml) on uptake of K^+ by both MTB and *M. smegmatis.* In support of an antagonistic effect of components of the bacteriological culture media on the anti-mycobacterial actions of the test naphthoquinones, it was also observed that the antimicrobial activities of these agents were strikingly superior when the bacteria were cultured in OADC-free Middlebrook medium. Clearly, added protein in the bacteria suspending medium antagonizes the antimicrobial actions of the test naphthoquinones, probably by binding of these agents. In the case of the growth studies, however,

additional interference by the other components of OADC (oleic acid, dextrose, catalase) cannot be excluded. Nonetheless, these mechanisms probably explain why the concentrations of the test naphthoquinones which caused complete (diospyrin and synthetic 7-methyljuglone) or almost complete (neodiospyrin) inhibition of K^+ uptake by the mycobacteria were lower than those which caused complete inhibition of bacterial growth. In addition, exposure of the bacteria to the test naphthoquinones in K^+ and glucose-free KONO medium prior to the addition of $^{86}Rb^+$, probably also increased their sensitivity to these agents.

The fact that MTB, unlike *M. smegmatis* retains its sensitivity to the test naphthoquinones in OADC-supplemented Middlebrook 7H9 broth in conventional MIC assays can be attributed to two possible reasons. Firstly, prolonged exposure of slow growing MTB to the test naphthoquinones in the MIC assay system, and secondly, MTB is more sensitive to these agents than *M. smegmatis*, as indicated by the differential effects of the naphthoquinones on mycobacterial energy metabolism as described below.

The effects of short-term (2 hours) exposure to the test naphthoquinones on the uptake of Ca^{2+} by *M. smegmatis,* as well as the effects of these agents on mycobacterial energy metabolism (MTB and *M. smegmatis),* were also measured. Exposure to the naphthoquinones at a fixed concentration of 1.5µg/ml did not result in influx of Ca^{2+}, suggesting that inhibition of K^+ influx is an early marker of altered membrane function, possibly due to selective inhibition of mycobacterial K^+ transporters, as opposed to non-

specific, membrane-disruptive activity. Nonetheless, a gradual, cumulative, membrane-disruptive effect of these compounds cannot be excluded.

Exposure of MTB to the test naphthoquinones, at the highest concentrations tested, was accompanied by significant decrease in microbial ATP levels, probably as a consequence of striking inhibition of K^+ transport and dissipation of the membrane potential. At lower concentrations of the test agents, significant increase in microbial ATP levels were observed, probably due to incomplete inhibition of K^+ transport and decreased utilization of ATP. On the other hand, exposure of *M. smegmatis* to either neodiospyrin or diospyrin resulted in dose-related increases in microbial ATP levels, with no inhibition detected, while, in the case of synthetic 7-methyljuglone, there is caused a significant decrease in ATP at the highest concentration tested, and increase at lower concentrations. These observations suggest, that *M. smegmatis* is less sensitive than MTB to the test naphthoquinones, and that longer exposure times; and/or higher concentrations of the test agents may be required to detect decrease in ATP levels. As mentioned above, the naphthoquinone-mediated increases ATP levels for *M. smegmatis* may be a partial consequence of inhibition of K^+ transport.

The above-mentioned findings clearly identify the mycobacterial membrane as a possible site of antimicrobial action of the test naphthoquinones, with mycobacterial K^+ transporters being particularly susceptible to these agents. Inhibitory effects on mycobacterial energy metabolism particularly in MTB, possibly as a secondary consequence of interference with K^+ transport with resultant alterations in membrane

potential, have also been documented. However, these may be difficult to detect over the relatively short exposure times used in the current study and may also be complicated by decreased consumption of ATP as mentioned above. Relatively few classes of antimicrobial agents target the bacterial membrane. Those which do, include the riminophenazines (Steel et al, 1999) and daptomycin (Steenbergen et al, 2009).

Although the results described in the current chapter have identified a possible mechanism of antimicrobial action of the test naphthoquinones, they also raise several issues with respect to efficacy in the therapeutic setting, especially the apparent limitation imposed by protein binding. Potential strategies to overcome these limitations, as well as the possible toxicity mentioned in chapter 4, may be to target these agents to MTB-infected pulmonary macrophages by direct administration into the airways using nanospray/nanoparticle technologies, with synthetic 7-methyljuglone appearing to show the greatest promise of 3 agents tested (based on MIC and ATP data).

The current study was undertaken with the primary objectives of characterizing the naphthoquinones, neodiospyrin and diospyrin from the roots of *Euclea natalensis*, as well as synthetic 7-methyljuglone, with respect to cytotoxicity and intracellular bioactivity against the H37Rv strain of MTB. Additional studies were also undertaken in an attempt to identify targets/mechanisms of the anti-mycobacterial activity of the test naphthoquinones, specifically effects on cation (K^+, Ca^{2+}) fluxes and microbial energy metabolism. *M. smegmatis* was also included in these mechanistic studies in order to confirm and extend the findings with MTB to another mycobacterial species.

The attempt to isolate the test naphthoquinones to an acceptable level of purity proved to be a challenging and time-consuming task. The compound they were first tested for their effects on two eukaryotic cell lines i.e. Vero cells (African green monkey kidney cells) and THP-1 cells (human acute monocytic leukemia cell line). All three test naphthoquinones were found to be cytotoxic for both cell lines in the low microgram concentration range (detectable from 1.5-12.5µg/ml). The effects of synthetic 7-methyljuglone on Vero cells had been described previously (van der Kooy, 2007) and were largely in agreement with the results of the current study. Importantly, the previously reported study (van der Kooy, 2007) and the current study, although both originating from the Department of Botany, University of Pretoria, were carried out completely independently in different laboratories. However, the effects of the test agents on THP-1 cells had not been described previously. These studies were of considerable importance as the THP-1 cell-line was used in subsequent experiments

designed to investigate the intracellular bioactivities of neodiospyrin, diospyrin, and synthetic 7-methyljuglone.

All three test agents were then tested for their activities against intracellular MTB *in vitro*, using experimentally infected, phorbol ester (PMA) – differentiated THP-1 cells as a model of MTB-infected human macrophages. Although all three test agents were found to be active against intracellular MTB, the concentrations of each agent required to cause complete suppression of bacterial growth were either close to (diospyrin and neodiospyrin) or higher (7-methyljuglone) than the MIC values for the H37Rv strain of MTB and close to those at which cytotoxic activity for eukaryotic cells was detected. The similarities between the MIC and intracellular bioactivity values for each agent suggest that they do not accumulate intracellularly to levels significantly higher than extracellular concentrations, and/or they do not complement the intrinsic antimicrobial systems of the THP-1 cell line. It is also possible that MTB-infected THP-1 cells may be more prone to the cytotoxic actions of the naphthoquinones, which in turn may counteract the intracellular bioactivity of these agents against this microbial pathogen.

The final stages of the laboratory research were devoted to characterizing the effects of the test naphthoquinones on: i) the uptake of K^+/Ca^{2+} by MTB (K^+ only) and *M. smegmatis;* and ii) microbial energy metabolism. None of the test agents caused influx of Ca^{2+} over the relatively short time course of these experiments. However, all three test agents at concentrations of 0.375µg/ml and upwards caused striking inhibition of the uptake of K^+ by both MTB and *M. smegmatis*, suggesting that these K^+ -transporting

systems may be primary targets of the naphthoquinones. An alternative scenario is that the naphthoquinones possess membrane destabilizing properties, with K^+ transport being particularly vulnerable, with decreased uptake of the cation being an early indicator of membrane dysfunction. Nevertheless, a direct effect of these agents on mycobacterial K^+ transporters cannot be excluded.

With respect to bacterial energy (ATP) metabolism, exposure of MTB to all 3 test agents, at the highest concentrations tested, caused significant decreases in microbial ATP levels, with synthetic 7-methyljuglone being the most potent. These inhibitory effects of the naphthoquinones on MTB energy metabolism may be secondary to interference with K^+ transport and dissipation of membrane potential. In the absence of the electrochemical gradient-induced proton motive force, the bacteria cannot synthesize ATP.

Somewhat surprisingly, the MIC values of all three test agents for both MTB and *M. smegmatis* were higher than those which caused complete inhibition of K^+ uptake, suggesting that interference with K^+ handling by the mycobacteria is not the only mechanism by which the naphthoquinones inhibit bacterial growth. However, given the critical requirement of K^+ for bacterial growth, this seems unlikely, and it is possible that the disconnect in terms of concentration effects of the naphthoquinones on bacterial K^+ uptake and growth may result from differences in the two assay systems. The most important of these are likely to be the 30 min pre-incubation period of the bacteria in K^+/glucose-free suspending medium which may render the bacteria particularly vulnerable to the naphthoquinones, as well as the absence of added protein.

Clearly, the identification of novel anti-TB agents is of paramount importance, given the public health threats posed by the HIV pandemic in developing countries and the related, ominous emergence of MDR/XDR strains of MTB. From an anecdotal/historical perspective, based on their use as "traditional remedies", *Euclea natalensis* –derived naphthoquinones most certainly merit intensive investigation as potential anti-TB agents. However, the results of the current study suggest that these agents may have a narrow therapeutic window because of the lack of a clear distinction between their cytotoxic and anti-mycobacterial activities, with synthetic 7-methyljuglone being the possible exception because of its low MIC for MTB and comparatively potent inhibitory effects on the energy metabolism of this microbial pathogen. The inhibitory effects of the test naphthoquinones on uptake of K^+ by MTB suggest that these agents, especially synthetic 7-methyljuglone, are of potential value in drug modelling, specifically the design of novel agents which selectively target mycobacterial K^+ transporters.

Appendix 1

Presentations

1. Invited as guest speaker to Conference on Annual Pharmaceutical conference AIDS, Malaria and Tuberculosis-North West University-Potchefstrrom-July 2007.

2. Poster presentation at Annual Faculty Day-Medical University of South Africa-Isolation of naphthoquinones from *Euclea Natalensis*-August 2008-Awarded first place for best poster presentation.

3. Presentation at Annual Physiological Conference-Cytotoxicity of naphthoquinones on THP-1 and Vero cell lines-September 2009.

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