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Antibiofilm activity of South African plant extracts against *Mycobacterium* spp. and their mechanism of action using Mycothiol reductase

By

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Submitted in partial fulfilment of the requirements for the degree

MSc: Medicinal Plant Science

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2015

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DECLARATION OF ORIGINALITY

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I Nomasomi Gasa hereby declare that this dissertation is my own original work. Where other people`s work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

Signature of student:..................................... Date......................................

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Acknowledgements

First and foremost I would love to thank Almighty God for being with me throughout and for being my anchor and pillar of strength.

I would also like to thank the following people, without whom it would have been impossible to complete this project:

My supervisor: Prof. Namrita Lall, for her guidance throughout the project and for granting me the opportunity.

Carel Oosthuizen, for being supportive for the entire study and for coming out of his way to help me. I am immensely grateful for your help and support.

My colleague, Anna-Marie Reid for always being so helpful and supportive.

Mabatho Nqephe for helping with the Cytotoxicity experiments.

My fellow students for making this a memorable experience.

Magda Nel and Elsa van Wyk for helping with the plant classification.

Jason Sampson for helping with the plant collection.

My daughter and family for being the source of my inspiration.

The National Research Foundation (NRF) and University of Pretoria for financial support.

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Abstract

Tuberculosis (TB) is an infectious disease, mostly affecting lungs or abdominal area. The causative agent of TB is a bacterium called *Mycobacterium tuberculosis*; it is the second leading cause of death in the world. Plants have been considered as a possible additive to current treatment, due to resistance of *M. tuberculosis* to commercially available drugs. Twenty South African medicinal plants ethanol extracts, tradititonally used to treat TB related symptoms were evaluated for their antituberculosis activity.

Twenty South African medicinal plant extracts were evaluated for *in vitro* antimycobacterial activities using the Microtiter Alamar Blue Assay and cytotoxicity with XTT (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) assay. *Mycobacterium tuberculosis* H37Rv strain was used as the test organism. Cytotoxicity test was done on human macrophages (U937 cell line). Of all the 20 extracts 7 showed activities against *Mycobacterium* with minimum inhibitory concentrations ranging from 125-31.25 µg/ml. *Ficus sur* had a selectivity index of 3 followed by *Salvia africana-lutea* and *Sphedamnocarpus pruriens* with selectivity indexes of 2.

The plant extracts were further tested for their activity against Glutathione disulfide reductase (Human analogue) and Mycothiol disulfide reductase (*Mycobacterium* analogue). The inhibitory activity of the plant extracts was determined using a DTNB-coupled Glutathione/Mycothiol disulfide reductase assay. *Typha minima* showed a potential inhibitory activity against Mycothione reductase (Mtr) with an effective concentration at which 50% activity is inhibited (EC_{50}) of 47.89±47.5µg/ml and had less inhibitory activity against Glutathione reductase (Gtr) with an EC_{50} of 813.5±3.21µg/ml.

The plant extracts were screened to evaluate whether they had antimicrobial and antibiofilm formation activity, utilizing *Mycobacterium smegmatis* as the test organism, which is a genetic homologue to *Mycobacterium tuberculosis*. No significant antimicrobial activity was observed from the plant extracts. Based on a visual analysis the plant extracts; *Sphedamnocarpus pruriens* (N), *Salvia africana-lutea* (L), *Withania somnifera* (R) showed considerable antibiofilm activity as compared to Ciproflaxicin (positive control) displayed a better inhibitory activity and was validated by determining quantitavely using crystal violet absorption. The effective concentration (EC_{50}) was determined and *Leonotis leonurus* L. (K), *Salvia africana-lutea* (L), and *Sphedamnocarpus pruriens* (N) showed potential biofilm

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formation inhibition at concentration 45.55±0.2475µg/ml, 100±56.83µg/ml and 61.39±60.59µg/ml respectively when compared to Ciproflaxicin, which inhibited at a concentration of 1.9802µg/ml.

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LIST OF ABBREVIATIONS

ATCC: American Type Culture Collection

CIP: Ciprofloxacin

DMSO: Dimethly sulfoxide (CH_2Cl_2)

DNA: Deoxyribonucleotide acid

EC_{50}: 50% Effective concentration

EDTA: Ethylene-diamene-tetra-acetic acid

ELISA: Enzyme-linked immunosorbent assay

FBS: Foetal bovine serum

GSH: Glutathiol

GSSG: Glutathione disulfide (oxidized)

Gtr: Glutathiol disulfide reductase

HEPES: (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid

HIV: Human immunodeficiency virus

IC_{50}: 50% inhibition concentration

INH: Isoniazid

MDR: Multiple drug-resistant

MIC: Minimum inhibitory concentration

MSH: Mycothiol

MSSH: Mycothione or Mycothiol disulfide

MTC: *Mycobacterium tuberculosis* complex

Mtr: Mycothiol disulfide reductase

NADPH: Nicotinamide adenine dinucleotide phosphate

OADC: Oleic acid. Albumin, dextrose, catalase

OD: Optical density

PANTA: Polymixin B, amphotricin B, nalidixic acid, trimethoprim, azlocillin

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PZA: Pyrazinamide

RIF: Rifampicin

TB: Tuberculosis

TM: Traditional medicine

WHO: World health organization

WWW: World Wide Web

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

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Chapter 1

Literature review, research question, aim

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1. Background

1.1 Traditional medicine

Plants play a pivotal role in the history of life on earth. Environment and human life is maintained by plants as plants provide food and oxygen. Most plants have different functions that are important to human health. Plants form the foundation of traditional medicine. Traditional medicine is associated with health practices, methods, knowledge and beliefs incorporating plant, animal and mineral-based medicines; spiritual therapies and exercises are all incorporated together to diagnose and heal in order to maintain well-being (Karou *et al*., 2007).

Traditional medicine is an essential part of the culture and traditions of African people. Most African countries have been using traditional medicines for centuries in which the remedies have been passed on from generation to generation. It has been estimated that approximately 80% of the population in developing countries depend on traditional medicine; it is the oldest medicinal system. People living in rural areas have limited access to conventional medicine and therefore use traditional medicine as it is more accessible and affordable. Common ailments such as coughs and high fevers have been treated for centuries by using traditional medicine. Scientists can identify plants that maybe medicinally useful through the help of traditional education. There are numerous recognized plant derived drugs which are currently being used by conventional medicine in contemporary hospitals all over the world for example morphine, digoxin, quinine, atropine, ergometrine and reserpine (Fennell *et al*., 2004; Karou *et al*., 2007; Light *et al*., 2005; Elujoba *et al*., 2005).

1.1.2 Traditional medicine and Ethnopharmacology

There are disadvantages which are related to traditional medicine, such as insufficient scientific proof of the phytochemicals present in the plants which may lead to negative side effects. The plants may have toxic compounds which may result in poisoning and death. Some people may have allergic reactions. Misdiagnosis can lead to the use of an improper plant, resulting in further complications in the patient. Dosages can be inaccurate leading to overdose of the remedy or toxic plants. No quality control measures are in place for traditional medicine (Elojuba *et al*., 2005; Fennell *et al*., 2004).

Ethnopharmacology is the scientific study of substances obtained from traditional medicines by different ethnic or cultural groups. Since most people use plants, ethnopharmacology is

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used to determine the safety and efficiency of the biologically active compounds isolated from traditional medicines. Scientific evaluation plays an important role in linking culture and science, by requiring the scientists to work with traditional healers. Ethnopharmacology has also linked different parts of science such as botany, pharmacology, chemistry and toxicology and revealed the significant role that traditional medicine has in primary health care. The interaction between traditional medicine and ethnopharmacology has resulted in new drug discovery (Raza, 2006).

1.2 South African plants

Southern Africa has approximately 25 000 higher plants of which 3000 plants species are used as medicines. The high number of medicinal plants is a result of the rich diversity that South Africa has as compared to the rest of the world. South Africa has a high number of indigenous plants, increasing the possibility of discovering or finding a cure for epidemic diseases in the country. Plants play a role in the economy as they contribute multi-million of rands by being traded in plant markets (Light *et al*., 2005; Lewu and Afolayan, 2009).

Alkaloids, Flavonoids, oils, terpenoids, phenolic acids, etc. are phytochemicals or biologically active secondary compounds found in plants and have different important functions in the human body. Most of these compounds have pharmacological properties. Twenty five percent of prescribed medicines come from plants or are derived from plant compounds. Plant species are rich source of secondary compounds which are responsible for the pharmacological properties of traditional medicines. A single indigenous species can contain multiple secondary compounds which can be used to act against microbes, bacteria and viruses (Kamatou *et al*., 2007).

1.2.1 South African plants and Ethnopharmacology

South African medicinal plants, traditionally used for ailments caused by microorganisms such as bacteria, microbes and viruses, need to be screened for active compounds. Screening is necessary as the acceptance of traditional medicine as an alternative form of health care is increasing.

There are many South African plants and their phytochemicals which have been reported to have antimicrobial activities, methanol extract of *Warburgia salutaris* inhibited the growth of *Escherichia coli* which means the above mentioned plant has antibacterial activity. Other

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plants such as *Bidens pilosa*, *Psidium guajava*, *Artemisia afra* also exhibited antibacterial activities (Rabe and van Staden, 1997).

Human immunodeficiency virus (HIV) is currently the most significant infectious pathogen with devastating consequences. South Africa represents the region of the world worst hit by HIV. Laboratory based investigations have sought to seek inhibitory properties of water and organic extracts, and isolated compounds against the essential enzymes of the virus. Powdered leaves of *Sutherlandia frutescens* subspecies *microphylla* (Fabaceae) have been reported to have the ability to improve the immune system, increase CD4 counts and decrease viral loads in AIDS patients. *Terminalia sericea*, *Bridelia micrantha* and *Lobostemon trigonus* have also been investigated for anti-HIV properties. *B. Micrantha and T. sericea* methanol extracts inhibited the polymerase and RNase functions with IC_{50} of 8.1µg/ml and 7.2µg/ml respectively. *L. trigonus* aqueous extract inhibited HIV-1 reverse transcriptase (Bessong and Obi, 2006).

Antimicrobial activities of plants: *Schotia, Cussonia* spp., *Halleria lucids* (leaves and stem), *Kigelia africana* and *Sclerocarya birrea* were investigated and found to have antimicrobial activities ranging from 0.15 to 3.00 mg/ml. *Usnea barbata* (lichens) has broad spectrum activity (0.1 mg/ml) against four Gram-positive micro-organisms, *Pentanisia prunelloides* showed highest activity (0.2 mg/ml) against *Staphylococcus aureus*. It has been reported that 3, 5, 7-Trihydroxyflavone (galangin) a compound found in *Helichrysum aureonitens* has antibacterial and antifungal properties ranging from 0.1 to 0.5µg/ml (Van Vuuren, 2008).

1.2.2 South African plants activity with respect to Tuberculosis

There are few South African plants that have been studied for antimycobacterial activity against *Mycobacterium tuberculosis*. These plants namely, *Cryptocarya latifolia*, *Thymus vulgaris, Nidorella anomala, Helichrysum melanacme, Croton pseudopulchellus, Ekebergia capensis, Polygala myrtifolia, Dodonea angustifolia, Galenia africana* and *Euclea natalensis* inhibit the growth of *Mycobacterium tuberculosis* (Lall & Meyer., 1999). These plants exhibited minimum inhibitory concentrations (MIC) lower than 50µg/ml. *E. natalensis* and *H.melanacme* were the most effective extracts against *M. tuberculosis* with MIC of 0.5 mg/ml (Lall & Meyer., 1999). The high antimycobacterial activity of *E.natalensis* is due to the presence of binaphthoquinoid compound; diospyrin and a naphthoquinone, 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone) which are active constituents

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responsible for the inhibitory properties of the *E. natalensis* plant roots showed activity at an MIC of 100µg/ml and 1.0µg/ml respectively (Lall & Meyer., 2001; Mahapatra *et al*., 2007). *Chenopodium ambrosioides*, *Ekebergia capensis*, *E. natalensis*, *H. melanacme, N. anomala* and *P. myrtifolia* were found to be inhibiting the *M. tuberculosis* resistant strain at 0.1mg/ml (Lall & Meyer., 1999). Four plants namely *Artemisia afra, Dodonea angustifolia*, *Drosera capensis* and *Galenia africana* showed activity against Mycobacterium smegmatis with concentrations ranging from 0.781-6.25mg/ml. The ethanol extracts of *D.angustifolia* and *G. africana* showed activity against *M. tuberculosis* at concentrations of 5.0mg/ml and 1.2mg/ml respectively (Mativandlela et al., 2008).

Acetone extracts of *Berchemia discolour* (12.5µg/ml), *Bridelia micrantha* (25µg/ml), *Warbugia salutaris* (25µg/ml) and *Terminalia seriacea* (25µg/ml) (Green *et al*., 2010). Recently 68 South African plants used to treat tuberculosis were screened for antimycobacterial activity. Of all the 68 plant extracts *Abrus precatorius* subsp. *africanus*, *Ficus sur*, *Pentanisia prunelloides* and *Terminalia phanerophlebia* showed potential activity against *Mycobacterium tuberculosis* (Madikizela *et al*., 2014). Plant species from the Rubiaceae family were screened for antimycobacterial activity against four *Mycobacterium* species: *M. tuberculosis, Mycobacterium smegmatis, Mycobacterium aurum* and *Mycobacterium bovis* BCG. Minimum inhibitory concentration values as low as 0.04mg/ml against *M. smegmatis* and *M. tuberculosis* were recorded (Aro *et al*., 2015).

1.3 The facts about Tuberculosis

Tuberculosis (TB) is an infection, mostly affecting lungs or abdominal area. The causative agent of TB is a bacterium called *Mycobacterium tuberculosis*; it is the second leading cause of death in the world specifically in Africa (WHO, 2011). In 2013, 9 million new infections occurred and 1.5 million people died due to TB. 95% of TB deaths occur in developing countries; TB is the third leading cause of death in women aged between 15-49 years. A person with active TB can infect 10-15 people in a year. Globally the incidence of TB infections has been dropping since 2006. The incidence rate (per 100 000 population) has been dropping by 1.3% annually since 2002. In 2013 the incidence, prevalence and mortality per 100 000 population in South Africa was 520, 590 and 38 respectively (WHO, 2014). South Africa is one of the 22 countries with the highest rate of TB infections; with KwaZulu-Natal province having the highest TB prevalence in the country. The good thing is that TB mortality has dropped by 40% since 1990 but this has not made a huge difference when

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compared to the population of people still infected with TB. This means there has been an improvement in controlling TB. Approximately 5-10% of the population infected with TB will develop active TB at a certain point in their lives. During latent infection, person has no symptoms and is not able to infect other people. The communities, health sector and families have been negatively affected by TB financially as well as emotionally (WHO, 2011, and 2014).

1.3.1 HIV and TB

HIV and TB co-infection have caused an impact on the treatment of both TB and HIV. People who are HIV positive and infected with TB are 21 to 34 times more prone to develop active TB than people who are HIV negative and infected with TB. There are 34 million people living with HIV worldwide and one third of them are infected with TB bacilli. It has been estimated that in 2013, 25% of people infected with HIV died from HIV-associated TB; in other words 360 000 people died of TB (WHO, 2014).

1.3.2 *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis complex (MTC) consists of different species namely: *M. tuberculosis*, *M. bovis*, *M africanum*, *M. microti* and *M. canetti*. MTC organisms cause TB and are pathogenic.They have characteristics which can be used to identify them. Firstly they are obligate aerobes that require a high oxygen content to grow, e.g. oxygen in the lungs. Secondly few are intracellular pathogens of animals and humans and have a generation time of 12 to 18 hours (Gen-probe, 2001).

1.3.3 Tuberculosis

The bacterium was first isolated by German physician Robert Koch in 1882. Tuberculosis mostly infects the lungs but can also affect other organs in the body such as bones, joints and the central nervous system. It is Gram-positive, rod-shaped (Figure 1.1) and had lipid rich cell wall which is impermeable to various simple drugs. As mentioned before it is a contagious disease, a person can get infected by inhaling a droplet of sputum or TB germs from the surrounding air if a person infected with TB coughs, sneezes, shout or spits he/she releases the saliva containing *Mycobacterium tuberculosis* (Medicine Net, 1996; WebMD, 2005).

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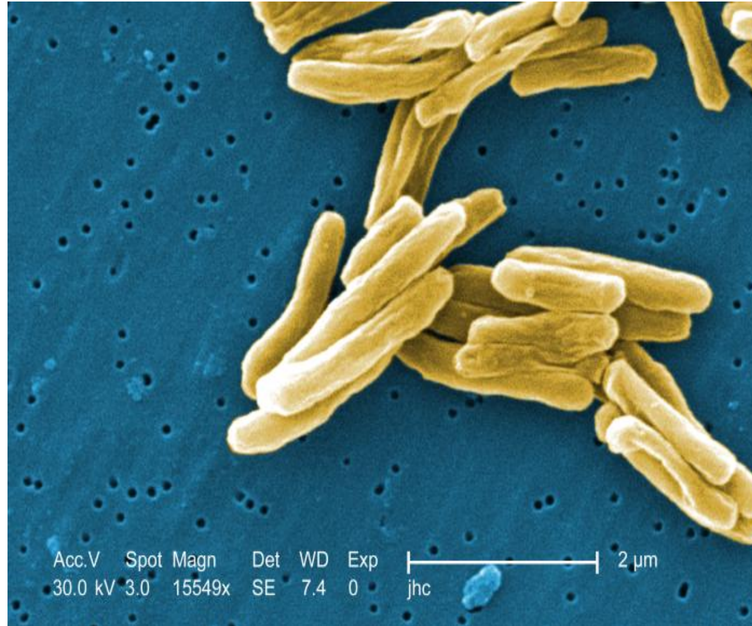

Figure 1.1: Microscope analysis of Mycobacterium tuberculosis (textbook of bacteriology, 2008).

The symptoms of tuberculosis

- Cough
- Night sweats
- Fever
- Weight loss
- Fatigue or weakness
- Diarrhoea
- Difficulty in breathing
- Chest pains
- Loss of appetite

First line drugs have been implemented that can suppress TB. Streptomycin was the first antibiotic to fight *Mycobacterium tuberculosis* and was introduced in 1946. Isoniazid originally was an antidepressant medication but became the second antibiotic to be introduced in 1952 (Figure 1.2). The above mentioned are first line drugs; together with Rifampin, Pyrazinamide and Ethambutol, all are used with other anti-TB drugs to inhibit the growth of *Mycobacterium tuberculosis*. Isoniazid and ethambutol inhibit cell wall synthesis. Pyrazinamide disrupts the plasma membrane and energy metabolism. Rifampin inhibits RNA

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synthesis. The disadvantages associated with these antibiotics are that drug resistance develops especially for Isoniazid and Streptomycin. It has been proposed that, depending on the condition of the patient, other alternative drugs, such as Rifapentine, Ethionamide, Cycloserine, Capreomycin, Levofloxacin and Moxiflixacin should be used (Livestrong, 2010).

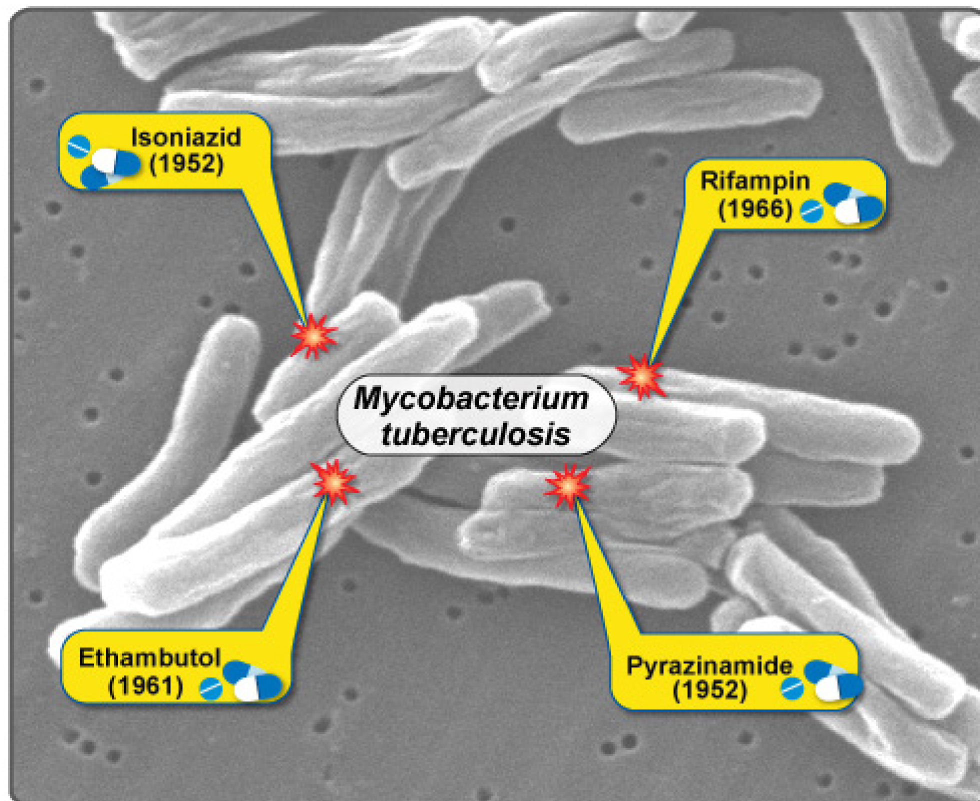

Figure 1.2: First line drugs for treatment of *Mycobacterium tuberculosis,* for drug sensitive TB (National Institute of Allergy and Infectious Diseases, 2007)

1.4 Multidrug resistance

Multi drug resistant (MDR) TB is caused by germs that are resistant to the normal antibiotics used to treat TB. MDR is caused by the patients not taking the medication correctly, as instructed or by using inappropriate drug combinations or patients not returning for medication. The disadvantage with MDR TB is that it is more difficult to treat as compared to normal TB because the TB bacilli are resistant to the normal medication. Therefore a second line of drugs (Figure 1.3) should be used; but they are less effective and have more side effects. It is stated that less than 50% of the people with MDR TB can be cured, and about 30% can die before the completion of the treatment (Western Cape government, 2012).

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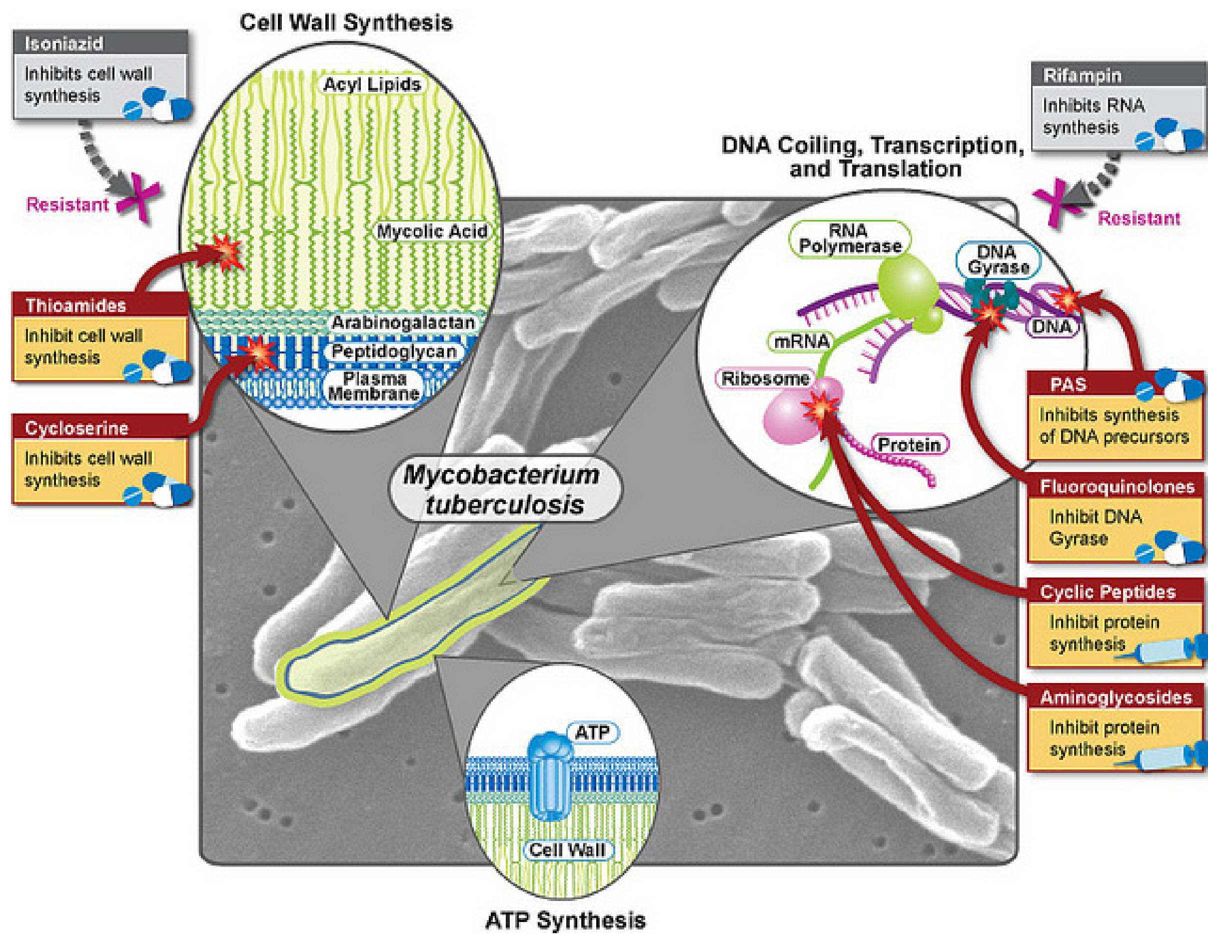

Figure 1.3: Multiple drug resistant TB and possible effective second line drugs (malnutritionandmalaria.blogspot.com, 2011)

1.4.1 Extensively drug resistant

Extensively drug resistant (XDR) TB is more severe than MDR because second line drugs are not effective against XDR-TB (Figure 1.4). The treatment options are very limited as it is resistant to both first-line and second line drugs (WHO, 2014).

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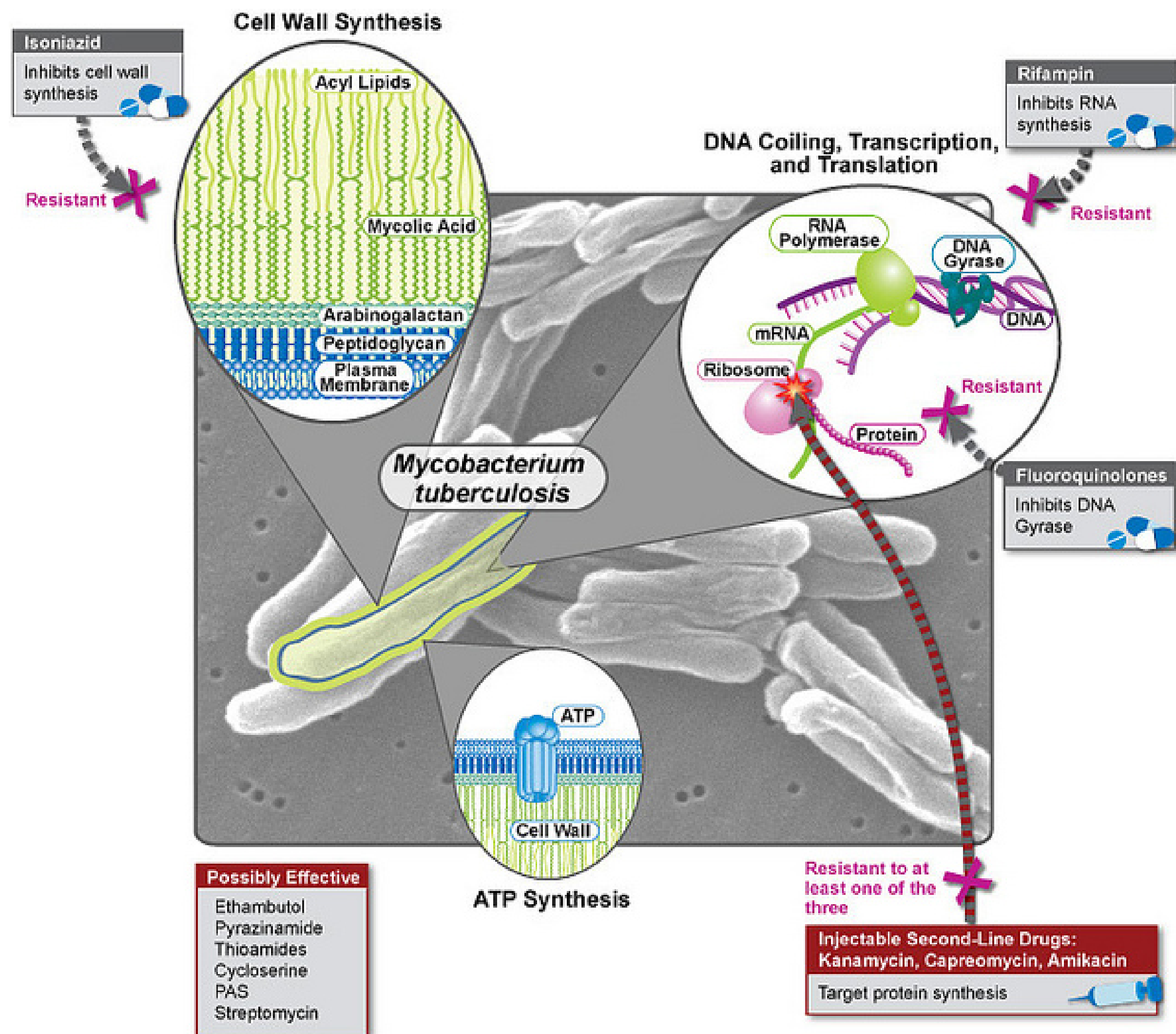

Figure 1.4: Extensively drug resistant TB (malnutritionandmalaria.blogspot.com, 2011).

1.5 Life cycle of *Mycobacterium tuberculosis*

The initial stage of infection is when *M. tuberculosis* bacilli are inhaled as droplet nuclei that have been exhaled into the atmosphere by an infected person. The nuclei are phagocytised by alveolar macrophages; this triggers a local inflammatory response which results in the invasion of subtending epithelium by the infected cells. This results in the recruitment of monocytes from the circulation and proliferation of blood vessels (Figure 1.5) of the infected site, providing fresh host cells for the expanding bacterial population (Russell *et al*, 2010).

The above mentioned cells are the main constituents of the granuloma which is responsible for the pathogenicity of this disease. The macrophages in the granuloma differentiate into several specialized cell types: multinucleated giant cells, foamy macrophages and epithelioid macrophages. A macrophage layer called fibrous cuff can form from the extracellular matrix

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material which results in the stratification of the granuloma. The disease is caused by the loss of blood vessels, increased necrosis and build up of caseum in the granuloma center. The granuloma is ruptured in the lungs and the infectious bacilli are released into the airways (Russell *et al*, 2010).

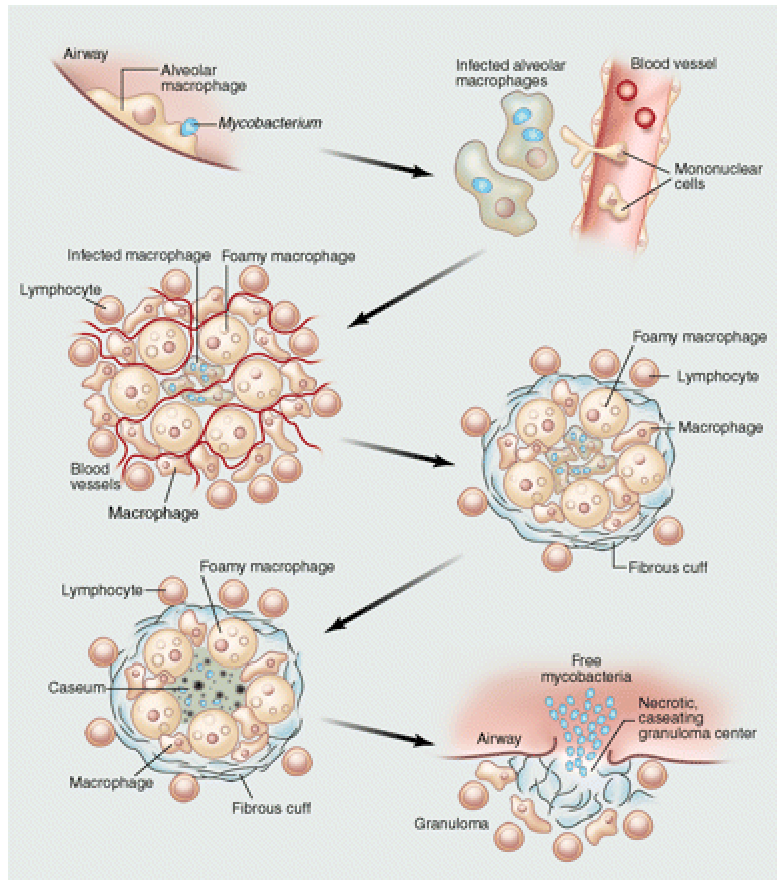

Figure 1.5: The life cycle of *Mycobacterium tuberculosis* (Russell *et al*., 2010).

1.6 Different mechanisms of action of TB drugs

First line drugs have different mechanism which they use to inhibit mycobacterial growth. Isoniazid (INH) is a prodrug and must be activated by a bacterial catalase-peroxidase enzyme called KatG present in *M.tuberculosis*. It inhibits the synthesis of mycolic acid, which is a long chain fatty acid-containing component of *M. tuberculosis* cell wall. Isoniazid is activated by KatG which is an enzyme with dual activities of catalase and peroxidase; it targets two enzymes which are involved in the elongation cycle of the fatty acid biosynthesis of *Mycobacterium* cell wall namely Enoyl-acyl carier protein reductase and β-ketoacyl-acyl carrier protein synthase which are believed to be the targets of the activated inhibitor (Lei *et al*., 2000).

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Rifampin is thought to inhibit bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase. It interferes with transcription by binding to the β subunit of RNA polymerase at a site adjacent to the RNA polymerase active centre and blocks RNA synthesis by physically preventing extension of RNA products beyond a length of 2-3 nucleotides (Hwang *et al*., 2003).

Pyrazinamide (PZA) is a prodrug that shortens treatment from the 9 to 12 months required to the current standard of 6 months. When it enters *M. tuberculosis* by passive diffusion it is converted to its active form Pyrazinoic acid (POA) by the bacterial enzyme pyrazinamidase (Pzase); POA has a pKa of 2.9 because it is an acid. POA is situated within the cell. Small amounts of protonated POA capable of diffusion across the membrane cause the collapse of the proton gradient therefore reducing membrane potential and affecting membrane transport in *Mycobacterium* (Shi *et al*., 2011).

1.7 Glutathione reductase and Mycothione reductase

Glutathione reductase and Mycothione reductase are enzymes which belong to the pyridine nucleotide-disulfide-reducing enzymes such as tyrpanothione reductase and thioredoxin reductase. They catalyze the pyridine-nucleotide-dependent reduction of a variety of substrates, including disulfide-bonded substrates such as mercuric ion, hydrogen peroxide and molecular oxygen. The above mentioned proteins maintain intracellular redox homeostasis to allow the proper functioning of a variety of biological processes such as cell cycle regulation, DNA synthesis and enzyme activity (Argyrou and Blanchard, 2004; Rawat and Av-Gay, 2007).

Glutathione reductase (GR) is a homodimeric flavoprotein which is found in pro- and eukaryotic organisms; an essential enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH) at the same time oxidizing reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction essential for the stability and integrity of red blood cells. This enzyme plays a pivotal role in the defence mechanism of the cell and protects it from the harmful effects of endogenous and exogenous hydroperoxides; it is an antioxidant enzyme (Argyrou and Blanchard, 2004).

Mycothione reductase (Mtr) is in the same enzyme family as GR which is mostly found in *Actinomycetes* and *Mycobacterium*. Mtr has homology with GR; it catalyzes the NADPH-dependent reduction of Mycothione to mycothiol. Mycothiol plays an essential role as an

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antioxidant in the actinomycetes; it is also produced in large amount in *M. tuberculosis*. Studies have been conducted in order to identify the enzymes of mycothiol biosynthesis as potential drug targets (Argyrou and Blanchard, 2004; Rawat and Av-Gay, 2007).

1.8 Biofilm

Microorganisms can form communities which adhere to biological and non-biological surfaces. These communities are known as biofilms which can be defined as aggregates of microbial cells that exist in close association with surfaces. It has been shown that these communities have tremendous impact on the local environment (Davey and O`toole, 2000). An interesting aspect about microbial communities is that they can be both beneficial and devastating for humankind and clinical environment respectively. Biofilms can develop into an almost-impossible-to-eradicate reservoir due to the variation in physiological status of microorganisms (Zambrano and Kolter, 2005). Their phenotypes can result in chronic bacterial infections such as *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients and they are thought to persist largely due to the formation of biofilms (Parsek and Singh, 2003; Namasivayam and Roy, 2013).

1.8.1 Biofilm formation

There is evidence of biofilm formation early in the fossil record, particularly in hydrothermal environments. Recent technology has shown that biofilms are not simple passive assemblages of cells stuck together but are rather structurally and dynamically complex biological systems. The initial attachment of bacteria to surface is often mediated by filaments that extend from the bacteria. There are proteinaceous appendages that act as anchors, transiently fixing the bacteria on the location. Once the bacterium is attached to the surface, biofilm-associated bacteria initiate the synthesis of an extracellular matrix (Figure 1.6) (Branda *et al*., 2005). The milieu (surroundings/environment) near the surface of the bacteria undergoes changes during biofilm formation. There can be variability in biofilm structure which could be due to numerous conditions such as constituents of microbial community, nutrient availability and surfaces (Biswas *et al*., 2011).

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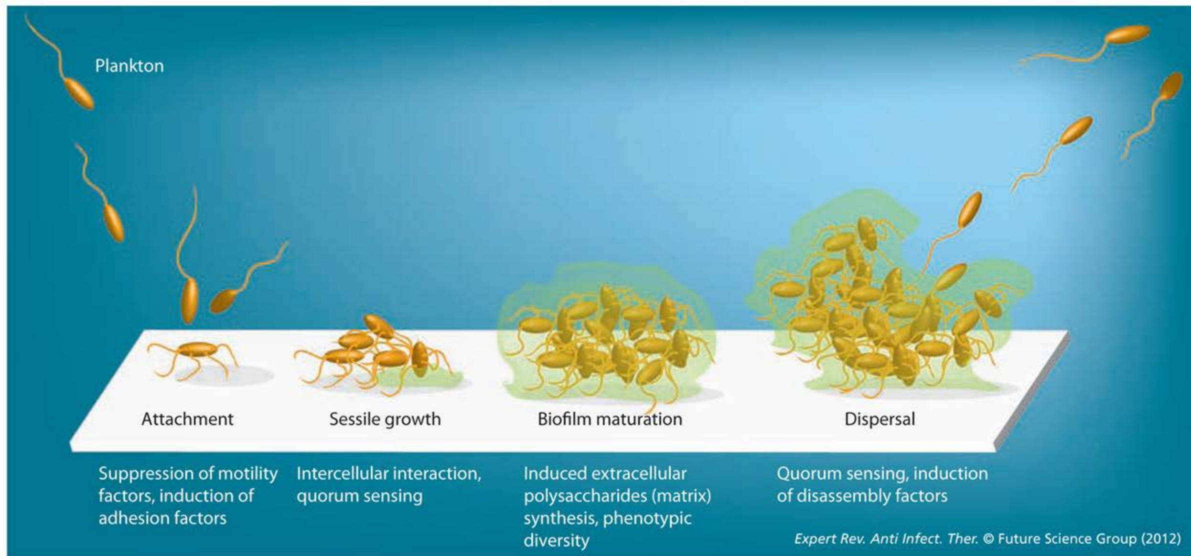

Figure 1.6: Schematic representation of biofilm formation (Islam *et al*., 2012)

There are different kinds of biofilm which can form for instance floating biofilm (also referred to as a pellicle) which forms on the surface of liquid culture media; the extracellular matrix constitutes secreted polysaccharides (exopolysaccharides), proteins and sometimes even nucleic acids. An interesting feature is that mycobacteria lack polysaccharides but are still able to form remarkable robust biofilms which either attach to hydrophobic solid surfaces or float as pellicles on the surface of liquid cultured media. It has been shown that the glycopeptidolipids of *Mycobacterium smegmatis* are important for initial surface attachment during biofilm formation (Recht *et al*., 2000; Zambrano and Kolter, 2005; Branda *et al*., 2005).

A class of fatty acids has been identified to play a pivotal role in the development of biofilm architecture in *Mycobacterium smegmatis.* It has been indicated that the synthesis of these fatty acids specifically involves GroEL1, a member of the chaperone proteins found in mycobacteria. Mycolic acids which are fatty acids with a very long chain (C_{70}-C_{90}), have been identified in the mycobacterial cell envelope and they are usually anchored to the envelope through covalent linkage. These mycolic acids provide a permeability barrier which is largely responsible for the abilities of these organisms to resist many common therapeutic agents (Zambrano and Kolter, 2005).

A study conducted by Ojha *et al*., 2005, shows that mycolic-acid profiles of *M. smegmatis* differ greatly between free-living bacteria and bacteria associated with biofilms. A new class of mycolic acids, much shorter (C_{56}-C_{68}) was formed by biofilm associated bacteria.

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1.8.2 Determination of Anti-biofilm formation activity

In the recent years antibacterial research has taken an anti-biofilm formation approach due to most bacteria using biofilm formation as a form of defence against drugs, leading to the bacteria being resistant to the drugs. Immense research is being done against biofilm formation, in connection with plants. Lemongrass (*Cymbopogon citrates* DC) oil showed antibiofilm activity against *Candida dubliniensis* at a concentration of 1.7mg/ml (Taweechaisupapong *et al*., 2012). Aquatic extracts of medicinal plants from the Brazilian xeric shrubland have been screened for antibiofilm activities against *Staphylococcus epidermis*. They showed activity at a concentration range of 0.4-4.0mg/ml (Trentin *et al*., 2013). *Azadirachta indica, Vitex negundu, Tridax procumbens* and *Ocimum tenuiflorumi* were tested against *Escherichia coli* biofilm. The biofilm was interpreted as follows; the first two plant extracts indicated 100% biofilm inhibition and the other two plant extracts indicated 60% biofilm inhibitions (Namasivayan & Roy, 2013). Plant derived natural compounds from *Krameria, Aesculus hippocastanum* and *Chelidonium majus* indicated antibiofilm activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* at an effective dose (EC50) of 24.5 & 15.2µM for *S. aureus* and 8.6 & 7.6µM for *S.epidermidis*.

South African plants have been screened for antibiofilm activity; *Mentha piperita* (Peppermint) has shown activity against *Pseudomonas aerugina*, *Candida albicans* & *Listeria monocytogenes* at an inhibition percentage of 38%, 28% and 50% respectively (Sandasi *et al*., 2010; Sandasi *et al*., 2011). *Rosmarinus officinalis* (Rosemary) and *Melaleuca alternifolia* were tested for inhibitory properties against *Listeria monocytogenes.* The plants inhibited biofilm formation by 50% (Sandasi *et al*., 2010). The Marula plant (*Sclerocarya birrea*) reduced biofilm formation of *Pseudomonas aeuginosa* by 75% (Sarkar *et al*., 2014).

Inhibition of biofilm formation by *Mycobacterium* species using plants extracts has been reported by Abidi *et al*., 2014. It was found that extract of *Azadirachta indica* (Neem) was efficient in reduction potential, which was 173% against *M.smegmatis* biofilm. Further analysis of biofilm inhibition with regards to cell-based phenotypic screening of *mycobacterium* has been done and led to the discovery of a small molecule TCA1. It has bactericidal activity against both drug-susceptible and resistant *M .tuberculosis* and can contribute to inhibition of biofilm formation (Wang *et al*., 2013).

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1.9 Project statement, aim and research question

TB is one of the leading killer diseases in the world: antibiotic treatment is less effective with multiple and extensively drug resistance occurring. Plants have been used for centuries as a source of medicine in most African countries. The pharmacological analysis of medicinal plants has shown that they have antimicrobial, antibacterial and few also have antimycobacterial activity.

South Africa has a rich and diverse flora with about 30 000 indigenous plants and 3000 of those are medicinal plants. A single plant can have many properties that are effective against different diseases.

AIM: To evaluate the efficacy of the selected South African plants as possible additives to tuberculosis treatment.

Research question:

- Can South African plants inhibit the growth of *Mycobacterium tuberculosis*?
- What are the mechanisms of action of *Mycobacterium* inhibitors?
- Can inhibition be achieved by targeting biofilm formation of the bacteria?

1.10 Methods of testing

- **Anti-mycobacterial activity**

Microtitre Alamar blue assay (MABA) was used to test the extracts activity against *Mycobacterium tuberculosis* H37RV strain.

- **Cytotoxicity**

To determine the toxicity of the plant extracts, differentiated U937 cells (macrophages) were used.

- **Enzymology/ mechanism studies**

Mechanism studies were conducted by evaluating the plants inhibition on Glutathione reductase and Mycothiol reductase enzymes.

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- **Biofilm inhibition**

Biofilm is used by *mycobacterium* as a defence mechanism against antibiotics and drugs; once biofilm forms the antibiotics and drugs are unable to penetrate or rather eradicate planktonic microorganisms. The tested plants in the study were evaluated for their inhibition on biofilm formed by *Mycobacterium smegmatis*, which is genetically homologues to *Mycobacterium tuberculosis*.

1.11 Dissertation layout

Chapter 1: Literature review & Research question

Chapter 2: Selection of medicinal plants and extract preparation

Chapter 3: Antimycobacterial activity of plant extracts against *Mycobacterium tuberculosis* and Cytotoxicity in U937 cell line

Chapter 4: Inhibitory effects of the selected medicinal plant extracts against Glutathione reductase and Mycothiol reductase

Chapter 5: Inhibition of biofilm formation by medicinal plant extracts against *Mycobacterium smegmatis*

Chapter 6: Overall conclusion and recommendations

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1.12 References

Abidi, S.H., Ahmed, K., Sherwani, S.K., Bibi, N., Kazmi, S.U., 2014. Detection of *Mycobacterium Smegmatis* Biofilm and its control by natural agents. International Journal of Current Microbiology and Applied Sciences 3 (4), 801-812.

Argyrou, A.,Blanchard, J.S. , 2004. Flavoprotein Disulfide Reductases: Advances in Chemistry and Function. In: Anonymous (Eds.), Progress in Nucleic Acid Research and Molecular Biology, Academic Press, pp. 89-142.

Bessong, P.O., Obi, C.L., 2006. Ethnopharmacology of human immunodeficiency virus in South Africa- a minireview. African Journal of Biotechnology 5 (19), 1693-1699.

Biswas,R., Agarwal,R.K., Bhilegaonkar,K.N., Kumar, A., Nambiar, P.,Rawat, S., Singh, M., 2011. Cloning and sequencing of biofilm-associated protein (bapA) gene and its occurence in different serotypes of Salmonella. Letters in Applied Microbioology 52 (2), 138-143.

Branda, S, S., Vik, A., Friedman, L., Kolter, R., 2005. Biofilm: the matric revisited. Trends in Microbiology 13 (1), 20-26.

Davey,M.E., O`toole, G.A., 2000. Microbial Biofilms: from ecology to molecular genetics. Microbiology and Molecular Biology Reviews 64 (4), 847-867.

Elujoba, A.A., odeleye, O.M., Ogunyemi, C.M., 2005. Traditional medicine development for medical and dental primary health care delivery system in Africa. African Journal of Traditional, Complementary and Alternative Medicines 2 (1), 46-61.

Fennell, C.W., Lindsey, K.L., McGraw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J., 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. Journal of Ethnopharmacology 94, 205-217.

Gen-probe.2001.Online.Mycobacterium tuberculosis complex culture identification test. available at: http://www.gen-probe.com/pdfs/pi/102896.pdf

Hwang, J.Y., Lee, C.W., Wu, M.C., Chang, H.M., 2003. Transacylation and de-esterification reactions of pectin as catalysed by pectinesterases from tomato and citrus. Journal of Agricultural Food Chemistry 51, 6287-6292.

Islam,M.S., Richards,J.P., Ojha, A.K., 2012. Targeting drug tolerance in mycobacteria: a perspective from mycobacterial biofilms. Expert Review of anti-Infective therapy 10(9), 1055-1066.

Kamatou, G.P.P., Van Vuuren, S.F., Van Heerden, F.R., Seaman, T., Viljoen, A.M. , 2007. Antibacterial and antimycobacterial activities of South African Salvia species and isolated compounds from S. chamelaeagnea. South African Journal of Botany 73, 552-557.

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Karou, D., Nadembega, W.M, C., Ouattara, L., IIboudo, D.P., Canini, A., Nikiema, J.B., Simpore, J., Colizzi, V., Traore, A.S., 2007. African ethnopharmacology and new drug discovery. Medicinal and Aromatic Plant Science and Biotechnology, 1-7.

Lall, N.,Meyer, J.J.M. , 1999. In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. Journal of ethnopharmacology 66, 347-354.

Lei, B., Wei, C.J., Tu., S.C., 2000. Action mechanism of antituberculosis isoniazid-activation *Mycobacterium tuberculosis* kat G, isolation and characterization of InhA inhibitor. Journal of Biological Chemistry 273, 2520-2526.

Lewu, F.B., Afolayan, A.J., 2009. Ethnomedicine in South Africa: The role of weedy species. African Journal of Biotechnology 8(6), 929-934.

Light, M.E., Stafford, G.I., van Staden, J., 2005. Riding the wave: South Africa`s contribution to ethnopharmacological research over the last 25 years. Journal of Ethnopharmacology 100, 127-130.

Livestrong.com. 2010. Online. Tuberculosis. Available at: http://www.livestrong.com/tuberculosis/ (Accessed 25 July 2014).

Madikizela, B., Ndhlala, A.R., Finnie, J.F., Van Staden, J., 2014. Antimycobacterial, anti-inflammatory and genotoxicity evaluation of plants used for the treatment of tuberculosis and related symptoms in South Africa. Journal of Ethnopharmacology 153, 386-391.

Mahapatra, A., Mativandlela, S.P.N., Binneman, B., Fourie, P.B., Hamliton, C.J., Meyer.J.J.M., Lall, N., 2007. Activity of 7-methyjuglone derivates against *Mycobacterium tuberculosis* and subversive substrates for mycothiol disulfide reductase. Bioorganic & Medicinal Chemistry 15, 7638-7646.

Malnutritionandmalaria.blogspot.com.2011.Online.Malnutrition,Malaria and the mycobacterium.Available at: http://malnutritionandmalaria.blogspot.com/2014/02/tuberculosis-first-line-treatment-of.html.

Mativandlela, S.P.N., Meyer, J.J.M., Hussein, A.A., Houghton, P.J., Hamilton, C.J., Lall, N., 2008. Activity against *Mycobacterium smegmatis* and *M.tuberculosis* by extract of South African medicinal plants. Phyototherapy research 22, 841-845.

Medicinenet.com.1996.Online.Tubeculosis.available at: http://www.medicinenet.com/tuberculosis/article.htm

Namasivayam, S. K. R., and Roy, E. A.,2013. Antibiofilm effect of medicinal plant extracts against clinical isolate of biofilm of Escherichia coli. International journal of pharmacy and pharmaceutical Researc 5(2), 486-489.

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National Institute of Allergy and Infectious Diseases. 2007. Online. Tuberculosis. Available at: http://www.niaid.nih.gov/topics/tuberculosis/Pages/Default.aspx (Accessed 30 July 2014).

Ojha, A., Anand, M., Bhatt, A., Kremer, L., Jacobs, W.R.,Hatfull, G.F., 2005. GroEL1: A Dedicated Chaperone Involved in Mycolic Acid Biosynthesis during Biofilm Formation in Mycobacteria. Cell, 1-13.

Parsek,M.R., Singh,P.K.,2003. Bacterial Biofilms: An emerging link to disease pathogenesis. Annual Review of Microbiology 57, 677-701.

Rabe, T.,van Staden, J. , 1997. Antibacterial activity of South African plants used for medicinal purposes. Journal of ethnopharmacology 56, 81-87.

Rawat, M., Av-Gay, Y., 2007. Mycothiol-dependent proteins in actinomycetes. Federation of European Microbiological Societies Microbiological reviews 31, 278-292.

Raza, M., 2006. A role for physicians in ethnopharmacology and drug discovery. Journal of Ethnopharmacology 104, 297-301.

Recht, J., Martinez, A., Torello,S., Kolter, R., 2000. Genetic analysis of sliding motility in *Mycobacterium smegmatis*. Journal fo bacteriology 182 (15), 4348-4351.

Russell, D.G., Barry, C.E., Flynn, J.L., 2010. Tuberculosis: What we don`t know can, and does, hurt Us. Science 328, 852-855.

Sandasi, M., Leonard, C.M., Van Vuuren, S.F., Viljoen, A.M., 2010.

Sandasi, M., Leonard, C.M., Van Vuuren, S.F., Viljoen, A.M., 2011. Peppermint (*Mentha piperita*) inhibits microbial biofilms *in vitro*. South African Journal of Botany 77 (2011), 80-85.

Sarkar, R., Chaudhary,S.K., Sharma, A., Yadav, K.K., Nema, N.K., Sekhoacha, M., karmakar, S., Braga, F.C., Matsabisa, M.G., Mukherjee, P.K., Sen, T.,2014. Anti-biofilm activity of Marula - a study with the standardized bark extract. Journal of Ethnophamacology 154, 170-175.

Shi, W., Zhang, X., Jiang, X., Yuan, H., Lee, J.S., Barry, C.E., Wang, H., Zhang, W., Zhang, Y., 2011. Pyrazinamide inhibits trans-translocation in *Mycobacterium tuberculosis*. Science 333 (6049), 1630-1632.

Taweechaisupapong, S., Ngaonee, P., Patsuk, P., Pitiphat, W., Khunkitti, W., 2012. Antibiofilm activity and post antifungal effect of lemongrass oil on clinical *Candida dubliniensis* isolate. South African Journal of Botany 78, 37-43.

Trentin, D.S., Silva, D.B., Amaral, M.W., Zimmer, K.R., Silva, M.V., Lopes, N.O., Macedo, A.J., 2013. Tannins possessing bacteriostatic effect impair *Pseudomonas aeruginosa* adhesion and biofilm formation. PloS one 8(6), 66257.

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Van Vuuren, S.F., 2008. Antimicrobial activity of South African medicinal plants. Journal of ethnopharmacology 119, 462-472.

Wang, F., Sambandan, D., Halder, R., Wang, J., Batt, S.M., Weinrick, B., Ahmad, I., Yang, P., Zhang, Y., Kim, J., Hassani, M., Huszar, S., Trefzer, C., Ma, Z., Kaneko, T., Mdluli, K.E., Franzblau, S., Chatterjee, A.K., Johnsson, K., Mikusova, K.,Besra, G.S., Futterer, K., Robbins, S.H., Barnes, Walker, J.R., Jacobs, W.R., Schultz., P.G., 2013. Identification of a small molecule with activity against drug-rsistant and persistent tuberculosis. Proceedings of the National Academy of Sciences 110 (270, 2510-2517.

WebMD.2005.Online.Lung disease and respiratory health centre for tuberculosis. Available at: http://www.webmd.com/lung/tc/tuberculosis-tb-topic-overview

Western Cape Government. 2012. Online. Tuberculosis. Available at: http://www.westerncape.gov.za/eng/your_life/4502 (Accessed 26 July 2014).

World health organization 2010, 2011, 2014 (www.who.com).

Zambrano,M.M., Kolter.R., 2005. Mycobacterial biofilms: a greasy way to hold it together. Cell 123 (5), 762-764.

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Chapter 2

Selection of the medicinal plants and extracts preparation

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2. Background

Twenty South African medicinal plants were selected for screening against *Mycobacterium* species. They were selected based on their traditional usage for treatment of various tuberculosis related symptoms such as chest/abdominal pains, fever and coughing. The aim of this chapter is to give more detailed information on the selected medicinal plants.

2.1 *Alectra sessiliflora* (Vahl) Kuntze var.sessiliflora

Figure 2.1: *Alectra sessiliflora* (Yellow witchweed) plant (zimbabweflora.co.zw, 2008)

Alectra sessiliflora from Orobanchaceae family is an erect annual herb (Figure 2.1), up to 60 cm tall with straight, simple or branched hairy stem which is indigenous to South Africa. Leaves are opposite, simple, no stipules, petiole up to 3 mm long, blade circular to ovate, margin entire to coarsely toothed and rigid-hairy to almost glabrous (Figure 2.1.1A). The flowers are solitary in axils of upper leaves; bisexual, slightly zygomorphic, sessile with pedicel, bracteoles linear to filiform, 5-lobed with triangular lobes; corolla campanulate and pale yellow to dull orange (Figure 2.1.1B). The fruits are globose capsules in which the seeds are linear to clavate and small (Burkill, 2000; Philcox, 1990).

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- **Species Identity**

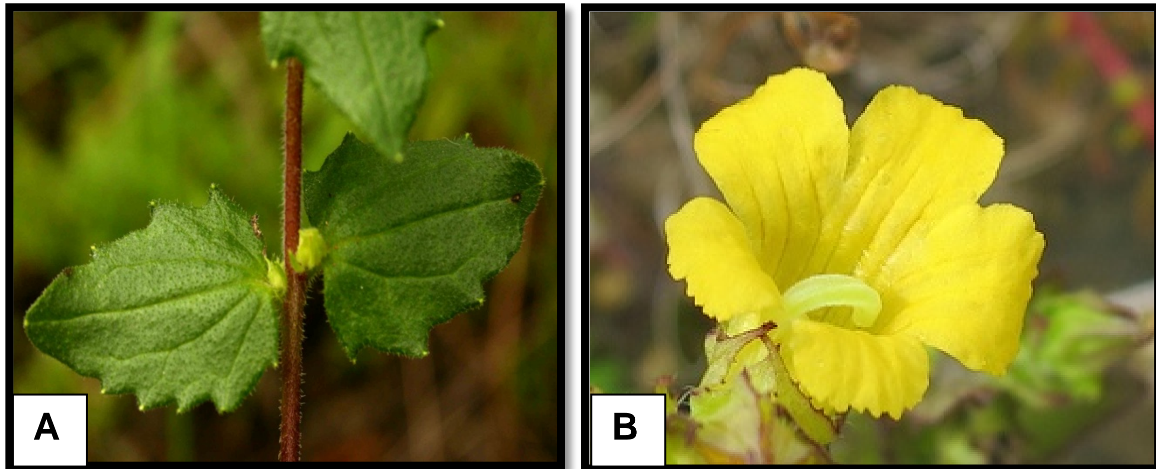

Figure 2.1.1: A: The leaves of *A.sessiliflora*; **B:** A flower from the *A.sessiliflora* plant (zimbabweflora.co.zw, 2008; ispotnature.org, 2014)

- **Distribution and Habitat**

Alectra sessiliflora is widespread and found all over tropical Africa and subtropical southern Africa and through the Indian Ocean islands to tropical Asia. It is indigenous to South Africa. It is found in grassland and cultivation areas (Burkill, 2000).

- **Traditional usage**

The root decoction is used as a mouthwash against toothache and given to younger children to treat diarrhoea. The fresh leaves are eaten by pregnant women as a galactagogue and the leaf decoction is used against kwashiorkor (Burkill, 2000).

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2.2 *Aloe plicatilis* (L.) Mill.

Figure 2.2: *Aloe plicatilis* tree (Fan Aloe) (plantzafrica.com, 2009)

- **Species Identity**

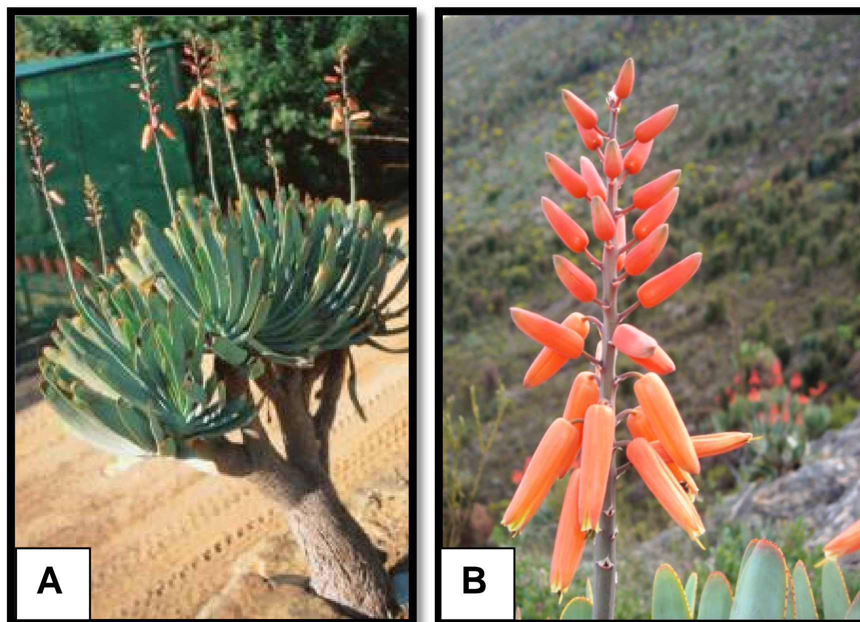

Figure 2.2.1: The Leaves and flowers of *Aloe plicatilis* (plantzafrica.com, 2009)

Aloe plicatilis (Asphodelaceae) commonly known as fan aloe is a much-branched shrub which may reach a height of 3-5 meters (Figure 2.2). The plant is comprised of dull grey-green, strap-shaped leaves positioned on a fork shaped stem (Figure 2.2.1A) in two opposite rows. The leaves have a clear sap. The racemes are cylindrical in shape and are always single

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in each leaf cluster. The arrangement of the leaves makes this aloe plant different from the rest in the genus. It flowers from August to October (plantzafrica.com, 2009).

- **Distribution and Habitat**

Aloe plicatilis is the only aloe tree restricted to the South-western Cape, where it grows from the mountains of Franschhoek to Elandskloof in the north. It grows mostly on steep rocky slopes and in high winter rainfall areas. It's one of the few aloe plants found in fynbos vegetation (Cousins, 2014).

- **Traditional usage**

Juice is used as a laxative and medicine (Grace *et al*., 2008; Van Wyk, 2008).

2.3 *Cassinopsis ilicifolia* (Hochst.) Kuntze

Figure 2.3: *Cassinopsis ilicifolia* (Lemon Thorn) (Hyde *et al*., 2014)

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- **Species Identity**

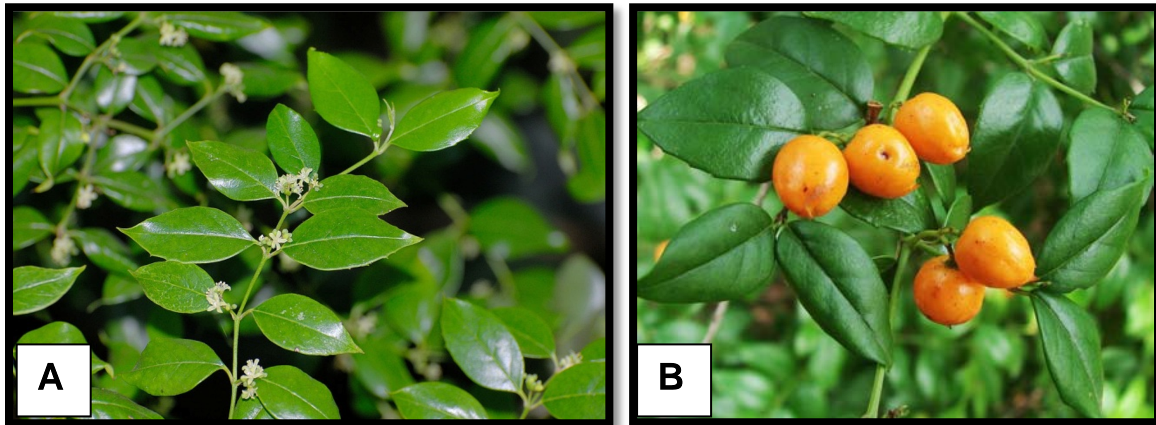

Figure 2.3.1: The leaves with flowers and fruits of *Cassinopsis ilicifolia* (petalfaire.co.za, 2014)

Cassinopsis ilicifolia (Icacinaceae) commonly known as Lemon Thorn is an evergreen scrambling shrub with a growth height of four meters (Figure 2.3). The maturity of the stems can be distinguished based on colour; young stems are glossy green while the older branches are brownish to pale grey. Composed of glossy green leaves (Figure 2.3.1A) above and dull green below (Hyde *et al.*, 2014). Can be identified by the Orange fruits which grow on them (Figure 2.3.1B).

- **Distribution and Habitat**

The lemon thorn is found in the southern and eastern Cape as well as in the eastern Free State, Lesotho, KwaZulu-Natal, Swaziland, Gauteng, Mpumalanga and Zimbabwe. It grows in the montane forest, riverine bush, on forest margins, in wooded kloofs and along streams (plantzafrica.com, 2014).

- **Traditional usage**

Used to treat stomach related ailments (Okem, 2011)

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2.4 *Dracaena aletriformis* (Haw.) Bos

Figure 2.4: *Dracaena aletriformis* (Dragon tree) (plantzafrica.com, 2003)

Dracaena aletriformis (Dracaenaceae) is a large-leaved evergreen, single stemmed small tree (Figure 2.4) with tan-coloured bark and yellow-green flowers. The leaves are glossy-green, leathery, strap-shaped and half drooping (Figure 2.4.1A). Orange berries (Figure 2.4.1B) can be found on these trees during summer (plantzafrica.com, 2003).

- **Species Identity**

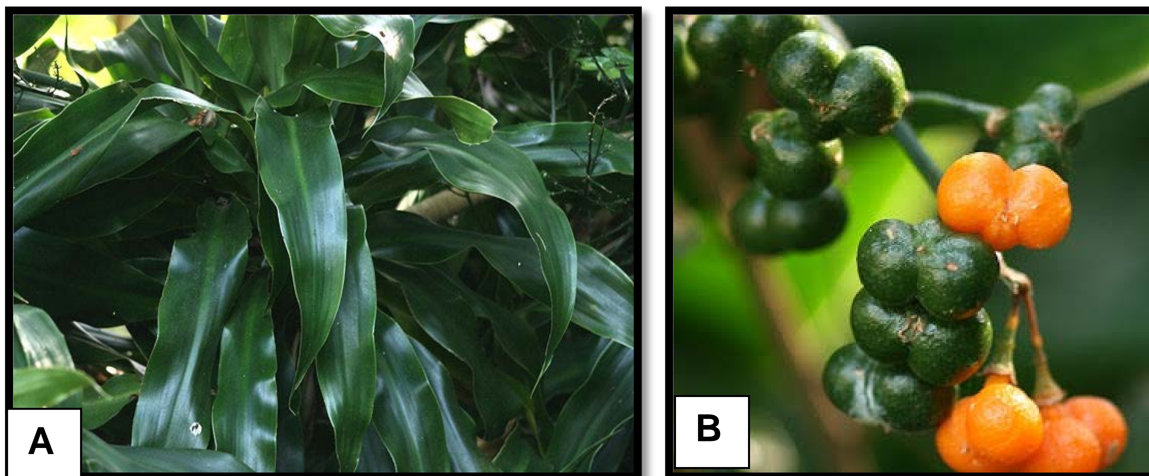

Figure 2.4.1: The leaves and fruits of *Dracaena aletriformis* (natureswow2.blogspot.com, 2013)

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- **Distribution and Habitat**

Dracaena aletriformis is distributed from Port Elizabeth to KwaZulu-Natal and into eastern and northern Gauteng. Frequently found in coastal dune forest and dry bushveld shades. Mostly found in humus-rich soil and dense stands (plantzafrica.com, 2003).

- **Traditional Usage**

Used as a wash to promote healing and stop bleeding also used to treat internal chest pains (plantzafrica.com, 2003).

2.5 *Dracaena draco*

Figure 2.5: *Dracaena draco* (Dragon tree) Plant (ascotvalegardencentre.com.au, 2014)

Dracaena draco from Dracaenaceae family is a monocot plant (Figure 2.5). The young plants have a single stem. At about 15 years the stem stops growing and produces the first flower spike, with white lily-like perfumed flowers (Figure 2.5.1). Followed by coral berries, a crown of terminal buds appear and the plant starts branching out. It is a slow growing plant, which can take up to 15 years to grow (Gupta *et al*., 2008).

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- **Species Identity**

Figure 2.5.1: The flowers of *Dracaena draco* (plantexplorer.longwoodgardes.org, 2014)

- **Distribution and Habitat**

It is found in rocks, cliffs, slopes and ravines (iucnredlist.org, 2014)

- **Traditional usage**

Dragons blood can be used for wound healing, coagulating, curing diarrhoea, lowering fevers, dysentery diseases, internal ulcers of mouth, throat, intestines and stomach, as an antiviral for respiratory and stomach viruses and for skin disorders such as eczema (Gupta *et al*., 2008).

2.6 *Eucomis autumnalis* (Mill) Chitt.subsp. *clavata* (Baker) Reyneke

Figure 2.6: *Eucomis autumnalis ssp. clavata* (Pineapple flower) Plant (bidorbuy.co.za, 2014)

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Eucomis autumnalis spp. clavata (Hyacinthaceae) can be described as a deciduous, summer growing bulb (Figure 2.6). The bulb forms a large rosette of leaves which are wavy-edged and soft in texture (Figure 2.6.1A). Cylindrical raceme inflorescence is crowded up with yellowish-green flowers (Figure 2.6.1B). Flowers are produced in summer (Leistner, 2000).

- **Species Identity**

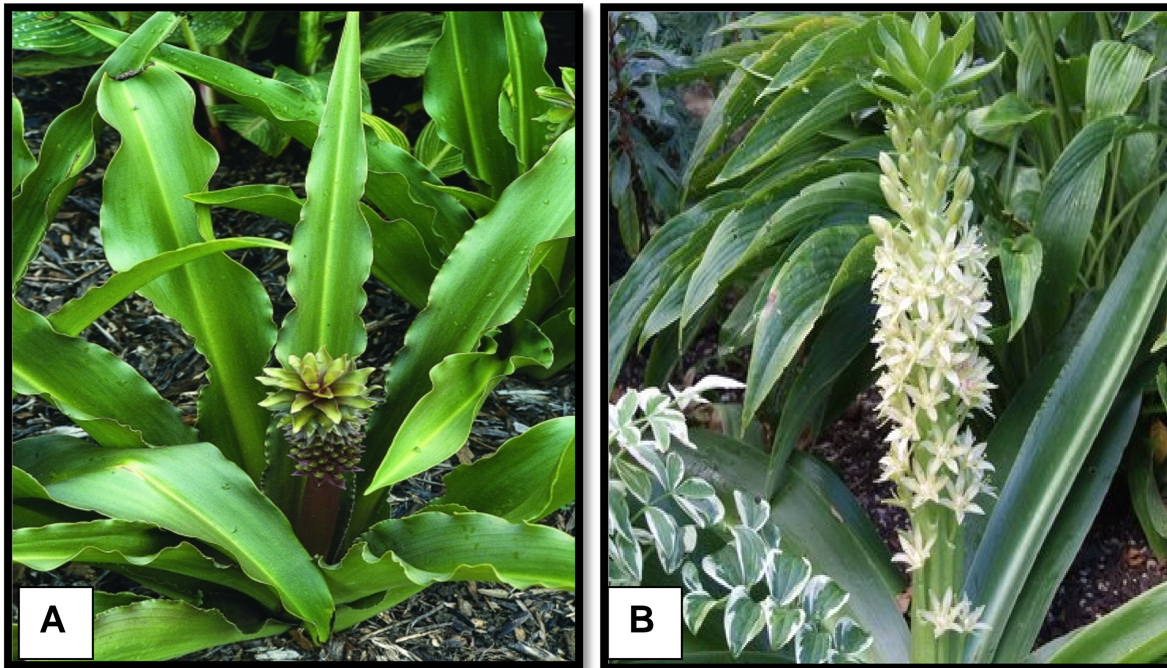

Figure 2.6.1: A: The leaves and; **B:** Flowers of *Eucomis autumnalis spp. clavata* (plantdelight.com, 2014; plantlust.com2014)

- **Distribution and Habitat**

Found in KwaZulu-Natal, Mpumalanga, Gauteng, North West, Northern Cape and Botswana. Mostly grows in open grasslands and marshes (Cheeseman, 2010).

- **Traditional Usage**

Decoctions of the bulb are used to treat low backache, stomach ache, fevers, coughs and respiratory ailments (Van Wyk *et al*., 1997).

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2.7 *Eucomis humilis* Baker

Figure 2.7: *Eucomis humilis* (dwarf pineapple flower) plant (pacificbulbsociety.org, 2014)

- **Species Identity**

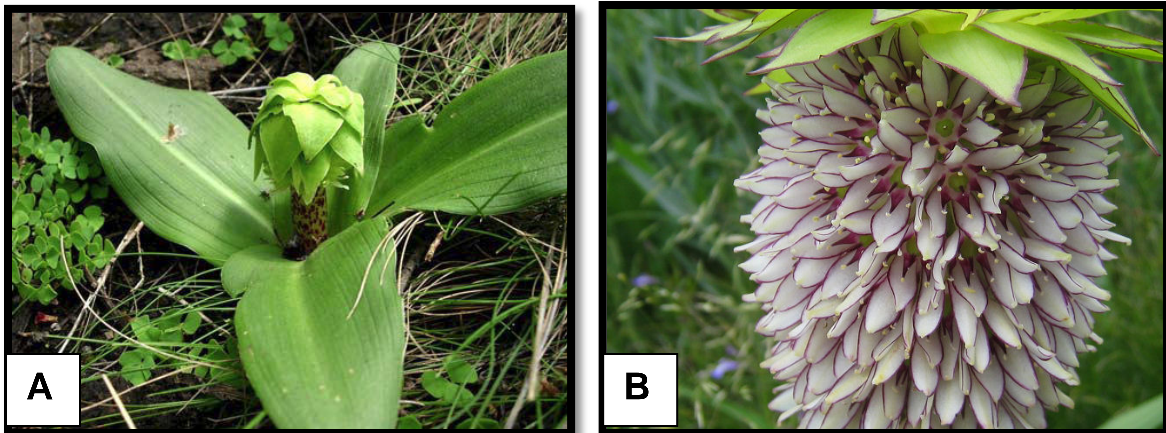

Figure 2.7.1: A: The leaves and; **B:** flowers of *Eucomis humilis* (pacificbulbsociety.org, 2014)

Eucomis humilis is a summer growing bulb which grows up to 400mm (Figure 2.7). Pineapple like inflorescences know as a cylindrical raceme and has keeled leaves (Figure 2.7.1A). Purple-tinted flower stems with greenish white flowers (Figure 2.7.1B). Bulbous plant flowers in summer (Leistner, 2000).

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- **Distribution and Habitat**

The dwarf pineapple flower is endemic to KwaZulu-Natal. Found growing in the alpine regions of the Drakensburg also in rocky stream gullies, wet rock overhangs and in grasslands (Borchers, 1996; Du Plessis & Duncan, 1989, Pooley, 2003).

- **Traditional Usage**

Used traditionally for the treatment of inflammation (Du Plessis & Duncan, 1989).

2.*8 Eucomis vandermerwei* I. Verd.

Figure 2.8: *Eucomis vandermerwei* (spotted-leaf eucomis) plant (growonyou.com, 2014)

Eucomis vandermerwei (Hyacinthaceae) is a dwarf alpine bulb with a distinct maroon colour and dark green leaves (Figure 2.8). They are summer-growing plants with perennial fleshy roots. The leaves are heavily spotted (Figure 2.8.1A) with deep maroon and have wavy margins with short raceme inflorescence (Figure 2.8.1B).

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- **Species Identity**

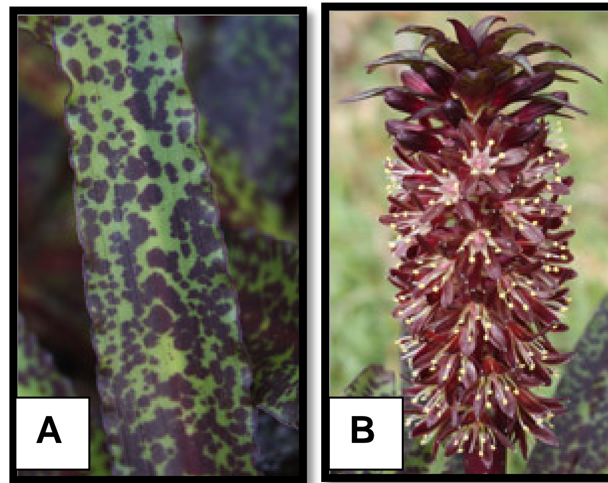

Figure 2.8.1: A: A blotched leaf of *E. vandermerwei*; **B:** The raceme inflorescent of *E. vandermerwei* (plantzafrica.com, 2014).

- **Traditional Usage**

Eucomis vandermerwei has been reported for anti-inflammatory properties (Plantzafrica.com, 2014).

2.9 *Euphorbia tirucalli* L.

Figure 2.9: *Euphorbia tirucalli* (rubber-hedge euphobia) plant (plantzafrica.com, 2014)

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Euphorbia tirucalli (Euphorbiaceae) is a multiple-branched, succulent plant about 3-5meters in height (Figure 2.9). The bark of older plants is grey with rough dents and ridges. It can be characterised with swelling on the bark and crosswise bands. The branches are cylindrical, smooth and glabrous-green. Yellow flowers are in clusters and fruits are divided in three parts (Figure 2.9.1) (plantzafrica.com, 2014).

- **Species Identity**

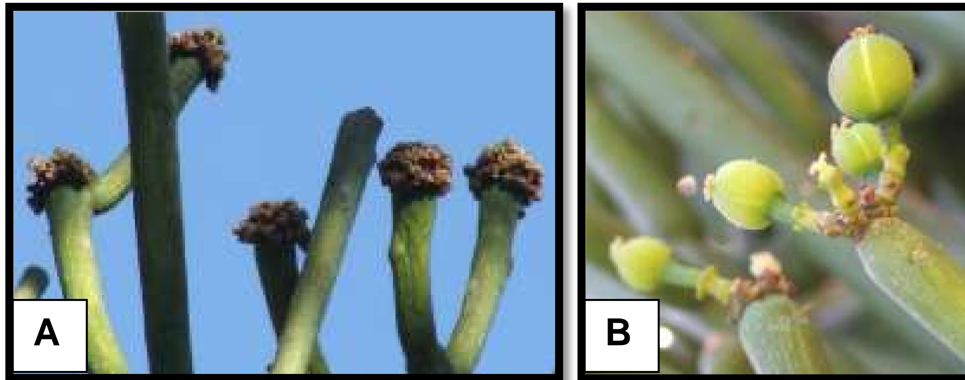

Figure 2.9.1: A: The flowers of *E. tirucalli*; **B**: The fruits of *E. tirucalli* (plantzafrica.com, 2014)

- **Distribution and Habitat**

Rubber-hedge euphorbia is found in the Eastern Cape and warmer parts of South Africa specifically frequent in KwaZulu-Natal (Van Wyk and Gericke, 2000).

- **Traditional usage**

The latex is used to treat sexual impotence, toothache, haemorrhoids, snake bites and cough (Gupta *et al*., 2013).

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2.10 *Faurea saligna* Harv.

Figure 2.10: *Faurea saligna* (Willow beechwood) tree (plantzafrica.com, 2009)

Faurea saligna (Proteaceae) commonly known as willow beechwood is a small, semi-deciduous tree about 7-10 m tall and can reach 20 m in certain areas such as in KwaZulu-Natal, it is indigenous to South Africa. The trunk is slender and straight or twisted and the bark is dark grey to black. The leaves (Figure 2.10.1A) are long, pointed, narrow, alternate, waxy and slightly sickle-shaped and droop downwards gracefully. The flowers occur in spring around September. The flower (Figure 2.10.1B) heads are formed in slender spikes, 120-150 x 20-30 mm. The flowers are green to cream white in colour, 12 mm long and have grey hairs when young. The fruit is moderately slow growing small nut with hairs (Palmer & Pitman., 1972; Rousseau, 1970; Van Wyk, 1984; Coates-Palgrave, 1988).

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- **Species identity**

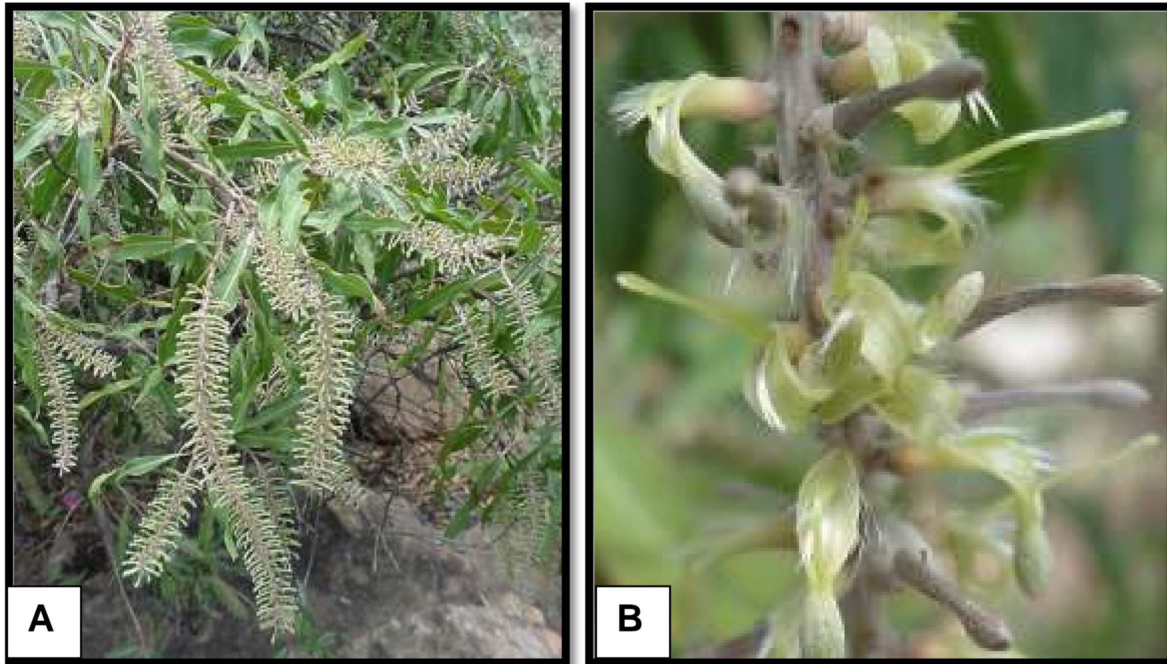

Figure 2.10.1: A: The leaves and; **B**: flowers of *Faurea saligna* (plantszafrica.com, 2009)

- **Distribution and Habitat**

Faurea saligna occurs at low-medium altitudes in open woodlands and on stony hillsides, sandy or red loamy soils and riverbanks. Mostly found in north-eastern parts of Southern Africa from Zimbambwe, Mozambique, Swaziland and South Africa specifically in North-west province, Gauteng, Limpopo and Mpumalanga (Coates-Palgrave, 1988).

- **Traditional usage**

The plant is used to treat diarrhoea epilepsy, bilharzias, helminthiasis (Hutchings, 1996; Chimponda and Mukanganyama, 2010)

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2.11 *Ficus sur* Forssk.

Figure 2.11: *Ficus sur* (broom cluster fig) tree (plantzafrica.com, 2009)

- **Species Identity**

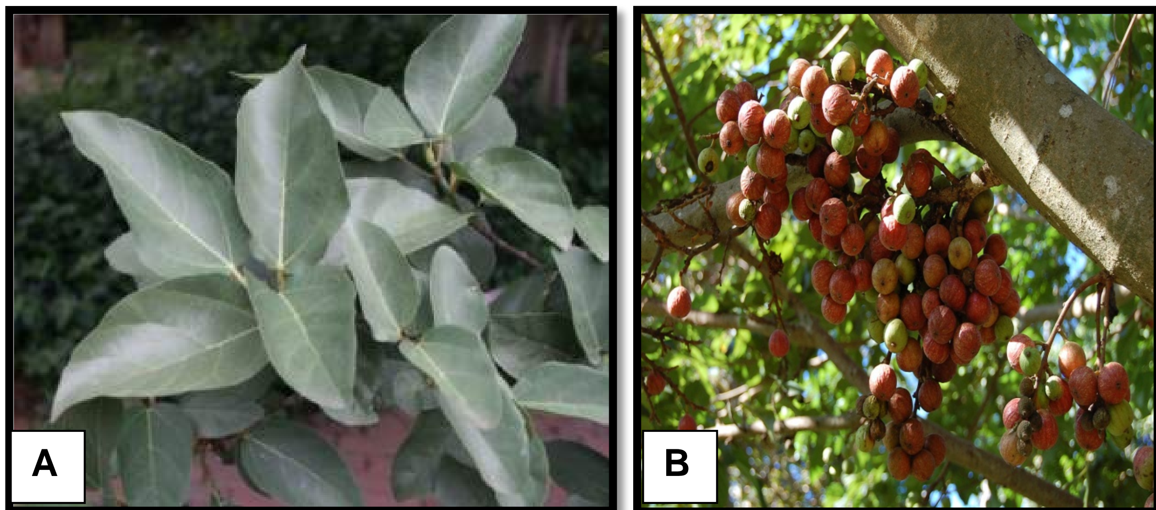

Figure 2.11.1: A: The leaves of *F.sur*; **B**: The fruits of *F.sur* (plantzafrica.com, 2009; zimbabweflora.co.zw. 2014).

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Ficus sur (Moraceae family) is a large, fast growing plant about 35 meters high (Figure 2.11) with oval green leaves (Figure 2.11.1 A). The species can be characterised by the fruits (figs) which are reddish in colour when ripe (Figure 2.11.1B). They bear in clusters low down in the trunk and can even arise at ground level at the roots (plantzafrica.com, 2009).

- **Distribution and Habitat**

Ficus sur is distributed from North Africa to Western Cape. Frequently found in temperate rainfall areas, near rivers and dry woodlands (plantzafrica.com, 2009).

- **Traditional usage**

Traditionally used to treat tuberculosis, influenza and colic (Watt & Breyer-Brandwijk, 1962; Hutchings, 1996; Eldeen & Van Staden., 2007)

2.12 *Ficus sycomorus* L. subsp. sycomorus

Figure 2.12: *Ficus sycomorus* (Sycamore fig) tree (prota4u.org, 2006)

Ficus sycomorus is a large savannah tree that can grow up to 20 meters in height (Figure 2.12). The bark of young stem is pale green whereas mature stem is grey-green, smooth with scattered grey scales. The leaves are egg-shaped and flat, with the narrow end at the base

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(Figure 2.12.1A). The figs are globose, yellow-red to reddish-purple when ripe and 3.5x5cm in size (Figure 2.12.1B) (Orwa *et al*., 2009).

- **Species Identity**

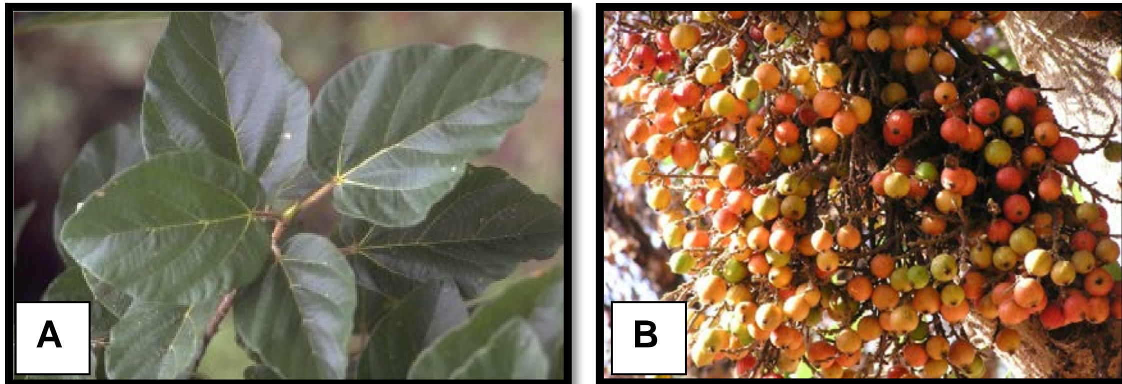

Figure 2.12.1: A: The leaves of *F.sycomorus*; **B**: The fruits of *F.sycomorus* (figweb.org, 2014; colorfulnature.com, 2014)

- **Distribution and Habitat**

Ficus sycomorus is found along streams, swamps, waterholes and rivers. It is found in afromontane rain forests, riparian woodland and rocky outcrops. Sycamore fig is native South Africa (Orwa *et al*., 2009).

- **Traditional usage**

The fruits are used to treat cough and burning chest inflammation (Lansky *et al*., 2008).

2.13 *Leonotis leonurus* (L.) R.Br

Figure 2.13: *Leonotis leonurus* (Wild dagga) shrub (plantzafrica.com, 2014)

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Leonotis leonurus from the lamiaceae family is a robust shrub which grows up to 2-3 meters (Figure 2.13). It has velvety stems and the leaves are narrow, long, rough above and velvety on the underside with saw-like edges (Figure 2.13.1A). The flowers are bright orange in compact clusters and whorls around the flower stock (Figure 2.13.1B) (plantzafrica.com, 2014).

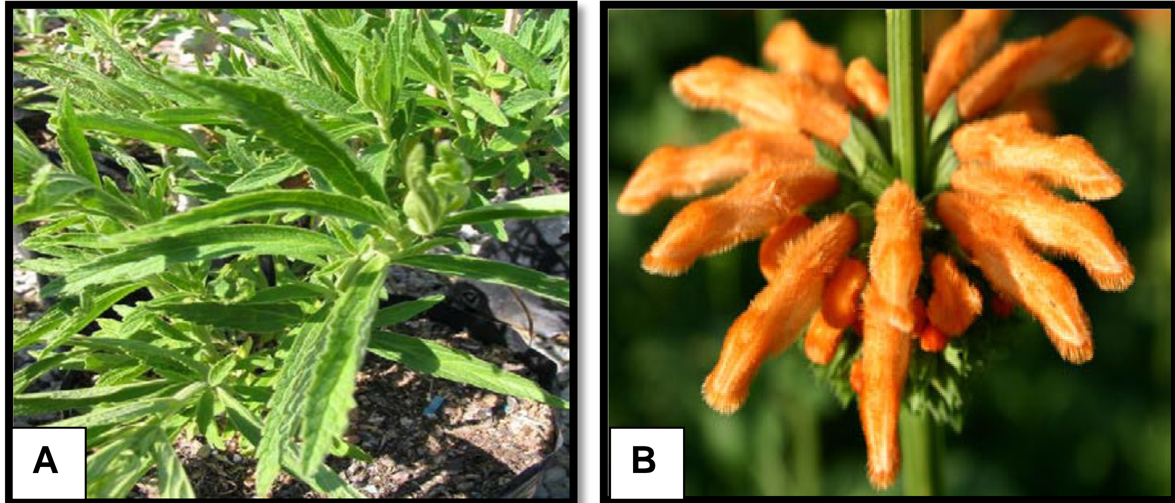

Figure 2.13.1: A: The leaves of *L. leonurus*; B: The flowers of *L. leonurus* (Mountain herb estate, 2014; bolokids.com, 2014).

- **Distribution and Habitat**

Leonotis leonurus is wide spread in South Africa and grows among rocks in grassland (plantzafrica.com, 2014).

- **Traditional usage**

The wild dagga is used to treat fevers, headaches, coughs and other conditions (plantzafrica.com, 2014).

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2.14 *Merwillia plumbea* (Lindl.) Speta

Figure 2.14: *Merwillia plumbea* (wild squill) plant (plantzafrica.com, 2014)

Merwillia plumbea (formely known as *Scilla natalensis*) from Hyacinthaceae family is a perennial bulb, with tall plumes of blue flowers (Figure 2.14). A rosette of 6 to 9 broad, narrowed leaves emerges from the top of the bulb (Figure 2.14.1A). The leaves have visibly distinctive veins, which give them a two-tone effect. Their colour is light green with grey-white overtones. The inflorescent is a many flowered slender raceme of bright violet blue (Figure 2.14.1B) (plantzafrica.com, 2014).

- **Species Identity**

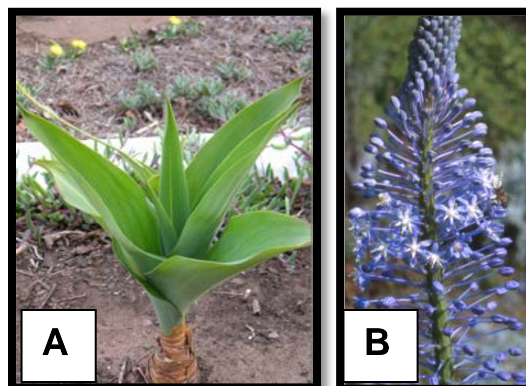

Figure 2.14.1: A: The leaves of *M. plumbea*; **B:** The flowers of *M. plumbea* (khumbulanursery.co.za, 2014; plantzafrica.com, 2014)

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- **Distribution and Habitat**

Merwilla plumbea is found in the eastern part of Southern Africa, all through the Eastern Cape, KwaZulu-Natal, Mpumalanga, Free State, Swaziland and Lesotho. It grows in sunny slope, rocky hills, cliffs, ledges, damp cliff faces, stream edges, near waterfalls and moist depressions. The Red Data list has been updated to vulnerable due to it being popular in KwaZulu-Natal muthi markets (plantzafrica.com, 2014).

- **Traditional usage**

The bulb of *M.plumbea* is used as a purgative, a laxative and for internal tumours. It is also used to treat chest pains and as an ingredient in a medicinal preparation for cattle suffering from lung infection (plantzafrica.com, 2014).

2.15 *Salvia africana-lutea* L.

Figure 2.15: *Salvia africana-lutea* (Beach salvia) shrub (plantzafrica.com, 2014)

Salvia africana-lutea is a hardy shrub, with distinctly coloured flowers (Figure 2.15). The flowers are reddish in colour and complemented by greyish-green aromatic foliage (Figure 2.15.1). It is a fast growing plant, growing up to 2 metres and is attractive to wild life (plantzafrica.com, 2014).

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- **Species Identity**

Figure 2.15.1: The flowers of *S. africana-lutea* (plantzafrica.com, 2014)

- **Distribution and habitat**

Salvia africana-lutea (Lamiaceae) is native to the Cape region of South Africa. It grows near the sea. The distribution stretches from the coast Namaqualand to the Cape Peninsula and eastwards to Port Alfred. Frequently found in coastal dune scrub and arid fynbos (plantzafrica.com, 2014).

- **Traditional usage**

Used as a tea to treat coughs, colds and bronchitis (plantzafrica.com, 2014).

2.16 *Sphedamnocarpus pruriens* (A. Juss.) Szyszyl.subsp.pruriens

Figure 2.16: *Sphedamnocarpus pruriens* plant (zimbabweflora.co.zw, 2014)

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Sphedamnocarpus pruriens from Malpighiaceae family is a deciduous climbing shrublet or climber with twining stems (Figure 2.16). The leaves are stalked with a round base (Figure 2.16.1A). Both surfaces of the leaf are densely covered with greyish hairs and a stalk that contains two glands in the upper half. The axillary umbels bear 2-6 bright yellow flowers, with wrinkled and short stalks (Figure 2.16.1B) (Van Wyk and Malan, 1998).

- **Species Identity**

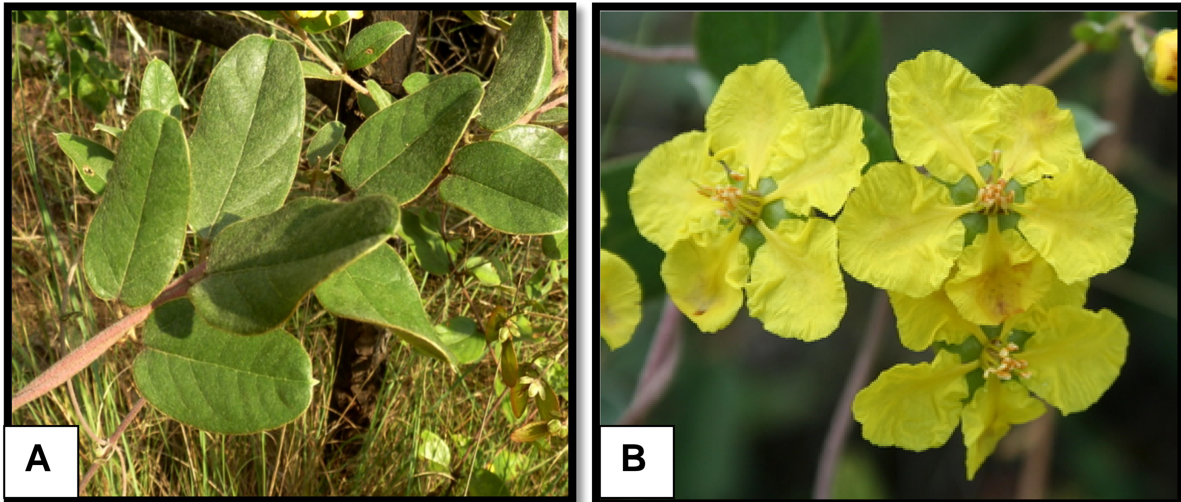

Figure 2.16.1: A: The leaves of *S.pruriens*; **B:** The flowers of *S, pruriens* (ispotnature.org, 2014)

- **Distribution and habitat**

Sphedomnocarpus pruriens is indigenous to South Africa and is widely distributed in Limpopo, North West, KwaZulu-Natal, Northern Cape, Mpumalanga and in countries such as Zimbabwe, Mozambique, Namibia and Botswana (zimbabweflora.co.zw, 2014).

- **Traditional usage**

The plant is traditionally used by the Zulu but no details are available. In Southern Rhodesia the plant is used as an African snake-bite remedy (Watt and Breyer-Brandwijk, 1962).

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2.17 *Tarchonanthus camphoratus* L.

Figure 2.17: *Tarchonanthus camphoratus* (camphor bush) plant (plantzafrica.com, 2014)

Tarchonanthus camphorates from Asteraceae family is a semi-deciduous small tree (Figure 2.17). The branches and foliage make a V-shaped canopy. The stem is covered with pale brown bark. The leaves are narrow and grey green above and pale grey and felted below (Figure 2.17.1A), with prominent venation on the bottom. The flowers are creamy-white in colour, in a branched inflorescence at the terminal end of the branch (Figure 2.17.1B). The fruits are covered with feathery cotton wool-like hairs (plantzafrica.com, 2014).

- **Species Identity**

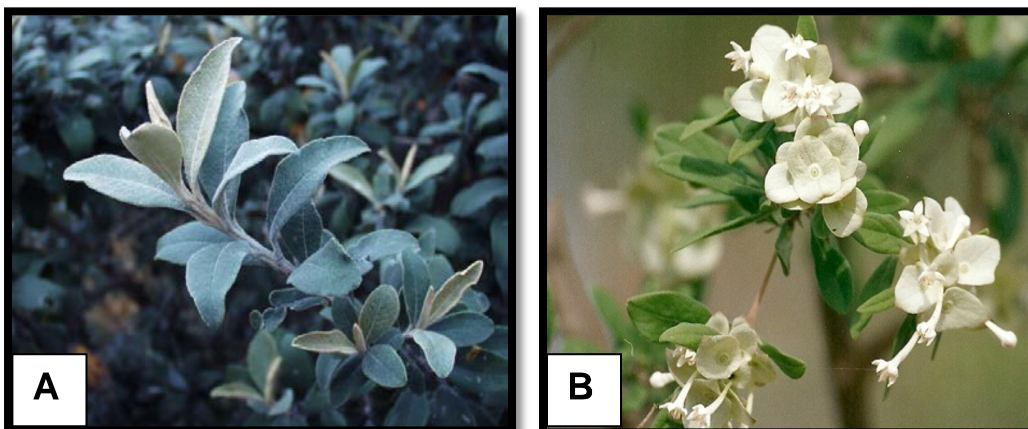

Figure 2.17.1: A: The leaves of *T. camphoratus*; **B:** The flowers of *T. camphorates* (bushtrucker.ch, 2014)

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- **Distribution and habitat**

The camphor bush grows in thickets of bushveld, grassland, forest and semi-desert in the Southern Africa. It grows predominantly in sandy soils in the low-lying and sand forest of the coast (plantzafrica.com, 2014).

- **Traditional usage**

The inhalation of smoke from the plant is used to treat headaches and blocked sinuses. Drinking boiled mixture of leaves is used to treat cough, abdominal pain and bronchitis (plantzafrica.com, 2014).

2.18 *Typha capensis* (Rohrb.) N.E.Br

Figure 2.18: *Typha capensis* (bulrush) plant (plantzafrica.com, 2014)

Typha capensis from Typhaceae family is a monoecious, perennial aquatic plant with creeping rhizomes (Figure 2.18). The stems are erect and simple, terminating in dense cylindrical flower-spikes. The leaves are long, bluish grey to green, strap-shaped and with parallel veins. The flowers are closely packed into a dense spike inflorescence, initially yellow and turning brown at a later stage (Figure 2.18.1) (plantzafrica.com, 2014).

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- **Species Identity**

Figure 2.18.1: The leaves and closely packed flowers (plantzafrica.com, 2014)

- **Distribution and habitat**

Typha capensis is found throughout the world and Southern Africa. It is predominantly found in aquatic areas, with slow-flowing waters. It is found in marshes, stream banks, dams and lakes (plantzafrica.com, 2014).

- **Traditional usage**

Used to improve circulation and for diarrhoea and dysentery. The rhizome decoction is used to treat venereal diseases (plantzafrica.com, 2014).

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2.19 *Typha minima*

Figure 2.19: *Typha minima* (Dwarf bulrush) plant (pfaf.org, 2014)

Typha minima from Typhaceae family are perennial herbaceous aquatic plants, with submerged overwintering buds (Figure 2.19). The stems are erect and simple, with blue-green, linear, very narrow and faint. These plants are monoecious, with mal e and female reproductive organs (Figure 2.19.1) on the same plant but packed into two separate inflorescences (Pignatti, 1982; Till-Bottraud *et al.*, 2010).

- **Species Identity**

Figure 2.19.1: The male and female reproductive structures of *T. minima* (pfaf.org, 2014)

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- **Distribution and habitat**

The plant *Typha minima* is found in Europe and Asia, in the rivers of the Alps and the Apennines and the Balkans and mountains of central Asia (Pignatti, 1982).

- **Traditional usage**

Typha minima pollen is used as an anticoagulant, diuretic, emmenagogue and haemostatic. It is also used to treat post-partum pains (pfaf.org, 2014).

2.20 *Withania somnifera* (L.) Dunal

Figure 2.20: *Withania somnifera* (winter cherry) plant (plantzafrica.com, 2014)

Withania somnifera from Solanaceae family is a small shrub, which grows up to 2 meters. The plant is covered with short, fine, silver-grey and branched hairs (Figure 2.20). The leaves are simple in an alternate arrangement, with entire to slightly wavy margins, broadly ovate and densely hairy below and green and hairless above (Figure 2.20.1A). The flowers are bisexual with yellow-orange stamens in multiples of 5 (Figure 2.20.1B). The fruit is hairless spherical berry, 5-8 mm across and orange-red to red when ripe (plantzafrica.com, 2014).

- **Distribution and habitat**

The winter cherry is widespread but not common in all provinces of South Africa, Namibia, Botswana, Lesotho and Swaziland. It is absent from the western parts of Northern and Western Cape Provinces. It grows in diverse types of vegetations in dry areas with high

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rainfall, coastal vegetation, grassland, Karoo, savannah, scrubland, woodland and thickets (plantzafrica.com, 2014).

- **Species Identity**

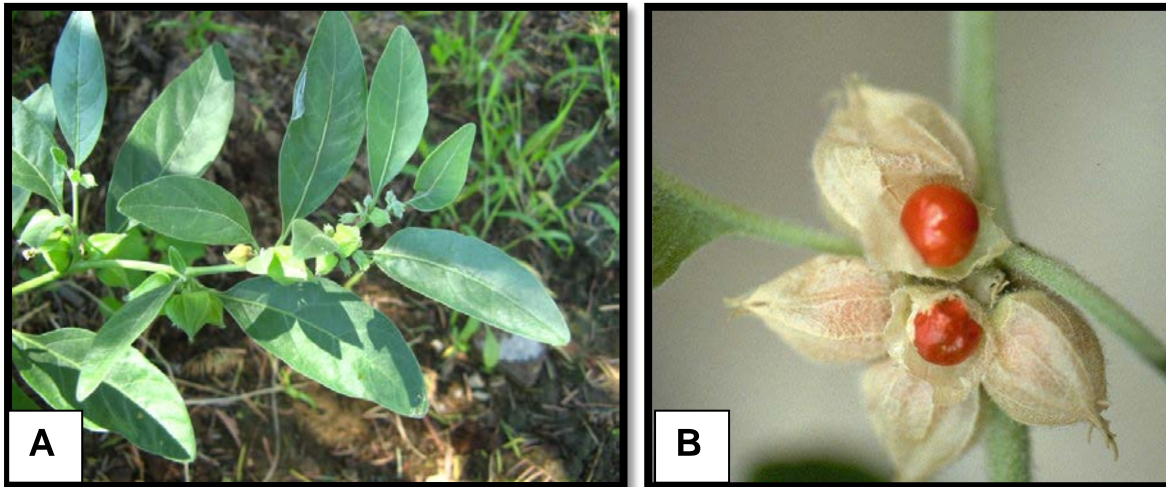

Figure 2.20.1: A: The leaves of *W.somnifera*; **B:** The fruits of *W.somnifera* (kyffhauser.co.za, 2014; flora.sa.gov.au, 2014)

- **Traditional usage**

The leaves are used to heal open and septic wounds, inflamed wounds, inflammation, haemorrhoids, rheumatism and syphilis. Infusion of the roots is drunk to treat fever (plantzafrica.com, 2014).

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3. Plant extraction

3.1 Plant Collection

Twenty South African medicinal plants were selected based on them being medicinal plants. The plant material was collected from the Manie van der Schijff Botanical Gardens at the University of Pretoria, with the help of the curator Jason Sampson. Authentication was established by submitting voucher specimens at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (Table 2.1).

Table 2.1: Plants selected for the current study

Plant extract	Plant part	PRU number/voucher number
***Alectra sessiliflora* (Vahl) Kuntze var.sessiliflora**	Roots	BC 52
***Aloe plicatilis* (L.) Mill.**	Leaves	119553
***Cassinopsis ilicifolia* (Hochst.) Kuntze**	Leaves and stems	119552
***Dracaena aletriformis* (Haw.) Bos**	Leaves	119554
***Dracaena draco* (L.) L.**	Leaves	119555
***Eucomis autumnalis* (Mill.) Chitt. subsp. *clavata* (Baker) Reyneke**	Leaves and flowers	119557
***Eucomis humilis* Baker**	Leaves	119558
***Eucomis vandermerwei* I. Verd.**	Leaves	119560
***Euphorbia tirucalli* L.**	Stems	119561
***Faurea saligna* Harv.**	Leaves	BC 37
***Ficus sur* Forssk.**	Fruits, leaves and stems	119566
***Ficus sycomorus* L. subsp. sycomorus**	Fruits, leaves and stems	119570
***Leonotis leonurus* (L.) R.Br.**	Leaves and stems	119569
***Merwilla plumbea* (Lindl.) Speta**	Leaves	119563
***Salvia africana-lutea* L.**	Leaves and stems	119562
***Sphedamnocarpus pruriens* (A.Juss) Szyszyl.subsp.pruriens**	Seeds and roots	BC 57
***Tarchonanthus camphorates* L.**	Leaves and stems	119564
***Typha capensis* (Rohrb.) N.E.Br.**	Leaves and roots	119565
***Typha minima* Funck**	Leaves and roots	119567
***Withania somnifera* (L.) Dunal**	Leaves and stems	119568

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3.2 Plant extracts preparation

The plant material was dried at 37ºC, and grinded into a powder. Ethanol solvent purchased from Merck (Pty) Ltd, South Africa was mixed with grinded plant material extract and vigorously shaken for 48 hours using a Labcon 3086U machine at moderate speed. Silva *et al*., 1998, used this method to ensure penetration of the solvent into the plant material and extraction of more compounds. The mixture was filtered through Whatman No.1 filter paper. Separation of the solvent from the plant extract was conducted using a rotator evaporator (Buchi R-200) in order to obtain a crude plant extract. The crude plant extracts were stored in pre-weighed, labelled glass Polytops at 7ºC.

4. Results and discussion

The plants selected for this study were from different families with 20% each selected from Hyacinthaceae, 10% each from the Dracaenaceae, Moraceaea, Lamiaceae and Typhaceae families respectively and 5% each from the rest of the plants (Figure 2.21).

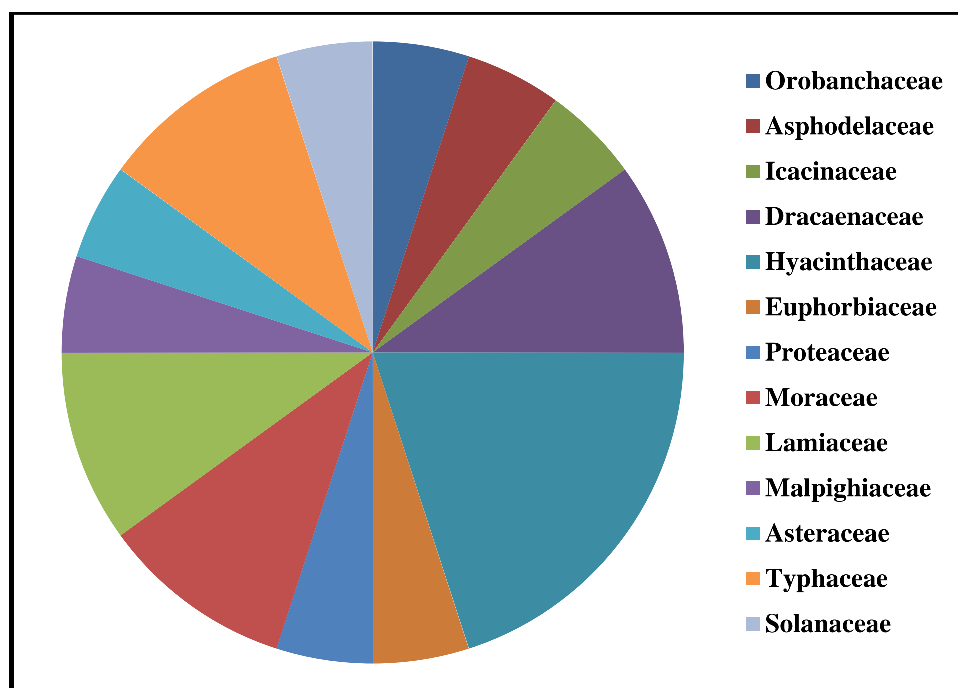

Figure 2.21: Selected plants from different plant families used in the study

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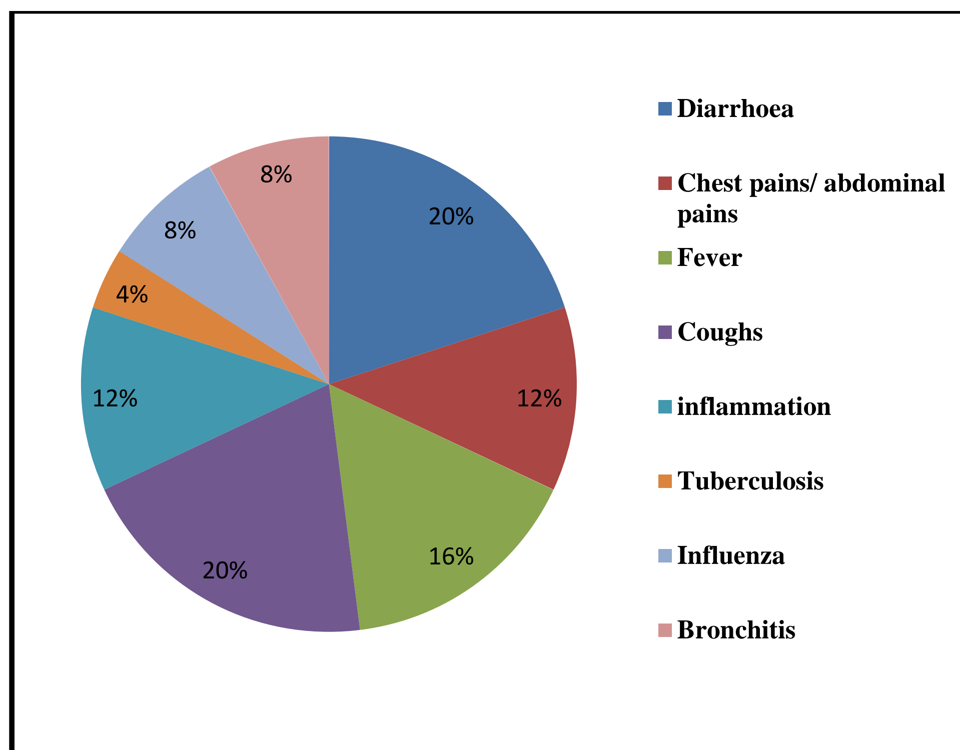

Figure 2.22: Various tuberculosis related symptoms treated using the selected plants

Figure 2.22 is a summary of ailments relevant to tuberculosis which are treated using the twenty selected plant samples. The plants are mainly used for the treatment of diarrhoea (20%), coughs (20%) and fever (16%).

The plant extracts were prepared using ethanol as an extraction solvent, which is a medium of polarity and is able to extract both polar and non-polar compounds. Figure 2.23 depicts the weights obtained after shade drying of the different parts of the plants and yields of the extracts.

The percentage yield for each plant was determined using the following formula:

$$\%\, yield = \frac{Final\ weight\ of\ extract}{Final\ weigth\ of\ powdered\ material} X100$$

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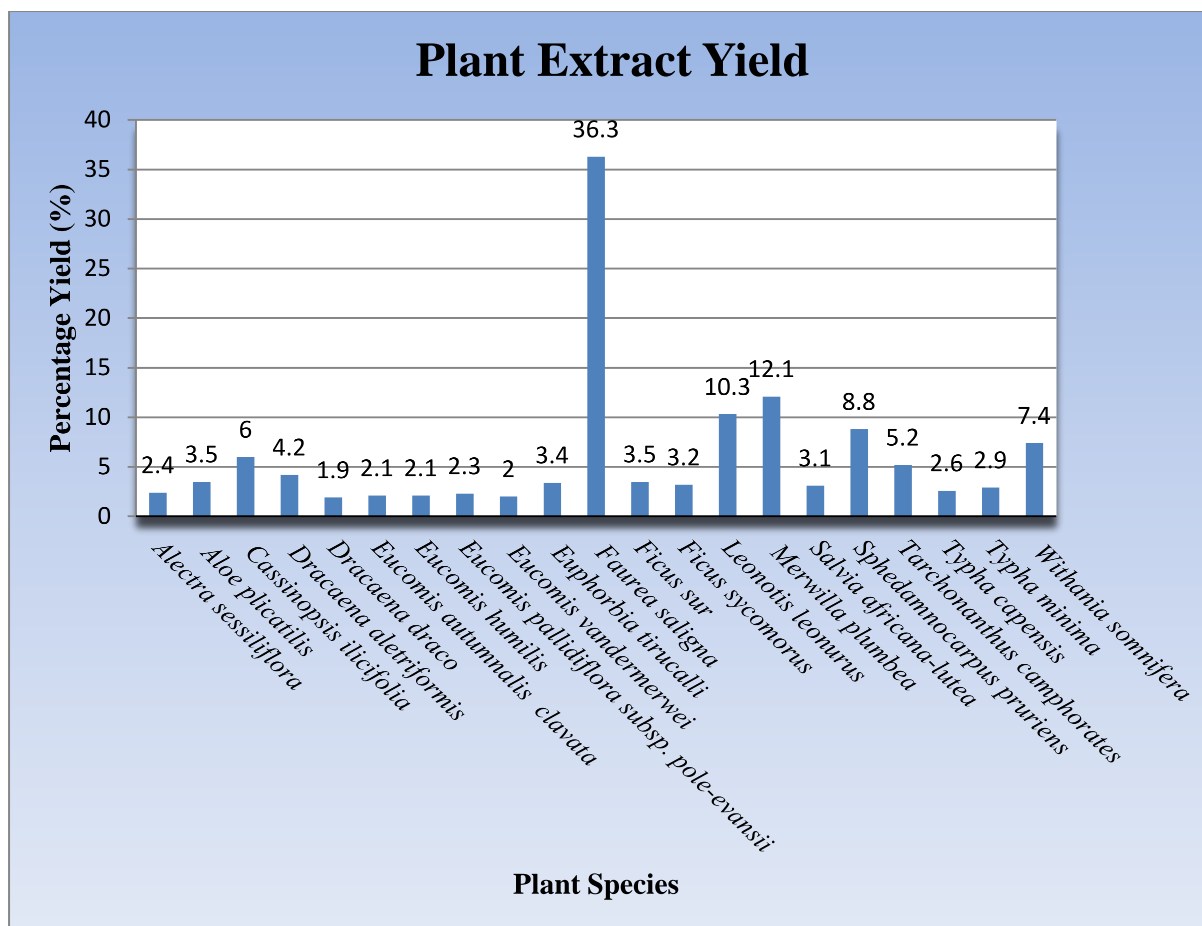

Figure 2.23: The percentage yield of th.e plant extracts used in this study

The percentage yield of the extracts differed among the plant families but plants from the same family had similar yield. *Eucomis* species gave the lowest percentage yield of an average of 2%. *Faurea saligna* gave the highest yield of 36.3%.

5. Conclusion

Medicinal plants from different plant families, collected from the University of Pretoria Botanical gardens, were selected based on their traditional use to treat tuberculosis related symptoms. The extracts were prepared successfully using ethanol solvent. The percentage yield of the extracts differed among the plant families.

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6. References

Ascot vale garden centre. 2014. Online. Dragon tree (*Dracaena draco*). Available at: http://www.ascotvalegardencentre.com.au/product/dragon-tree-dracaena-draco/ (Accessed 15 November 2014)

Bid or buy. 2014. Online. *Eucomis autumnalis* ssp. *clavata.* Available at: http://www.bidorbuy.co.za/item/22479647/10_Eucomis_autumnalis_Seeds_Pineapple_Flower_Pineapple_Lily_Wildepynappel_Indigenous_Bulbs_Seeds.html (Accessed 30 November 2014).

Bolo kids.com. 2014. Online. Leonotis leonurus (Lamiaceae). Available at: http://www.bolokids.com/index.cfm?md=Content&sd=Articles&ArticleID=60 (Accessed 25 November 2014).

Borchers, H., 1996. Greening the KwaZulu-Natal midlands. National Botanical Institute, Pretoria.

Burkill, H.M., 2000. The useful plants of West Tropical Africa second edition. Royal Botanic gardens, Kew, Richmond, United Kingdom, pg 686.

Bush trucker. 2014. Online. Shrubs and bushes. Available at: http://www.bushtrucker.ch/html/shrubs-bushes.html (Accessed on 18 November 2014).

Cheesman, L., 2010. *In vitro* bulb induction in *Eucomis zambesiaca* Baker. Doctoral dissertation.

Chimponda,T., Mukanganyama, S., 2010. Antimycobacterial activities of selected medicinal plantsfrom Zimbabwe against Mycobacterium aurum and Corynebacterium glutamicum. Tropical Biomedicine 27 (3), 595-610.

Coates-Palgrave, M., 2002. Coates Palgrave trees of Southern Africa, third edition. Struik publishers, Cape Town.

Colorful nature.com. 2014. Online. *Ficus sycomorus* L.. Available at: http://www.colorfulnature.com/picture.jsp?lang=en_US&category=flowers&picGroupId=Ficus_Sycomorus-m (Accessed on 21 November 2014).

Cousins, S. R., 2014. The ecology of *aloe plicatilis*, a tree aloe endemic to the Cape fynbos, South Africa, Doctoral dissertation.

Du Plessis, N., Duncan, G., 1989. Bulbous Plants of Southern Africa, A guide to their cultivation and propagation.Tafelberg, Cape Town.

Eldeen, I. M. S., Van Staden, J., 2007. Antimycobacterial activity of some trees used in South African traditional medicine. South African Journal of Botany 73(2), 248-251.

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Figweb. 2014. Online. *Ficus sycomorus* Linnaeus. Available at: http://www.figweb.org/Ficus/Subgenus_Sycomorus/Section_Sycomorus/Subsection_Sycomorus/Ficus_sycomorus_sycomorus.htm (Accessed 21 November 2014).

Flora of South Africa. 2014. Online. *Withania somnifera*. Available at: http://www.flora.sa.gov.au/efsa/lucid/Solanaceae/Solanaceae%20species/key/Australian%20Solanaceae%20species/Media/Html/Withania_somnifera.htm. (Accessed on 19 November 2014).

Flora of Zimbabwe. 2008. Online. *Alectra sessiliflora*. Available at: http://www.zimbabweflora.co.zw/speciesdata/species.php?species_id=151840 (Accessed 25 October 2014).

Flora of Zimbabwe. 2014. Online. *Ficus sur* Forssk.. Available at: http://www.zimbabweflora.co.zw/speciesdata/image-display.php?species_id=120400&image_id=10 (Accessed on 20 November 2014).

Flora of Zimbabwe. 2014. Online. *Sphedamnocarpus pruriens*. Available at: http://www.zimbabweflora.co.zw/speciesdata/species.php?species_id=133640 (Accessed on 28 November 2014).

Grace, O. M., Simmonds, M. S. J., Smith, G. F., Van Wyk, A. E., 2008. Therapeutic uses of *Aloe* L. (Asphodelaceae) in southern Africa. Journal of ethnopharmacology 119(3), 604-614.

Growonyou.com. 2014. Online. *Eucomis vadermerwei*. Available at: http://www.growsonyou.com/photo/slideshow/220662-eucomis-vandermerwei-octopus (Accessed on 30 November 2014).

Gupta, D., Bleakley, B.,Gupta, R. K., 2008. Dragon's blood: botany, chemistry and therapeutic uses. Journal of ethnopharmacology 115(3), 361-380.

Gupta, N., Vishnoi, G., Wal, A., Wal, P., 2013. Medicinal value of Euphorbia tirucalli a review. Research and Reviews: Journal of Pharmacognosy and Phytochemistry 1(2013), 16-25.

Hutchings, A., 1996. Zulu medicinal plants: an inventory. University of Natal Press.

Hyde, M.A., Wursten, B.T., Ballings, P., Palgrave, M. **,** 2014**.** Online. Flora of Zimbabwe: Species information: individual images: *Cassinopsis ilicifolia*.. Available at : http://www.zimbabweflora.co.zw/speciesdata/imagedisplay.php?species_id=137240&image_id=1 (Accessed 10 November 2014).

iSpot share nature. 2014. Online. *Alectra sessiliflora*. Available at: http://www.ispotnature.org/species-dictionaries/sanbi/Alectra%20sessiliflora (Accessed 12 November 2014).

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Khumbula indigenous garden. 2014. Online. *Merwilla plumbea*. Available at: http://kumbulanursery.co.za/plants/merwilla-plumbea (Accessed on 29 November 2014).

Lansky, E. P., Paavilainen, H. M., Pawlus, A. D., Newman, R. A., 2008. *Ficus* spp.(fig): Ethnobotany and potential as anticancer and anti-inflammatory agents. Journal of Ethnopharmacology 119(2), 195-213.

Leistner, O. A. 2000. Seed Plants of southern Africa: families and genera. Strelitzia 10.

Longwood gardens. 2014. Plant explorer: *Dracaena draco*. Available at: https://plantexplorer.longwoodgardens.org/weboi/oecgi2.exe/INET_ECM_DisplayPlant?ACCNUM=1988-1014*A (Accessed 15 November 2014).

Mountain herb estate. 2014. Online. Plant information: *Leonotis leonurus*. Available at: http://www.herbgarden.co.za/mountainherb/herbinfo.php?id=76 (Accessed on 25 November 2015).

Nature World of Wonder 2. 2013. Online. Large-leaved dragon tree (*Dracaena aletriformis*). Available at: http://natureswow2.blogspot.com/2013/09/large-leaved-dragon-tree-dracaena.html (Accessed 15 November 2014).

Okem, A., 2011. Pharmacological Activities of Selected South African Medicinal Plants. University of KwaZulu-Natal, Pietermaritzburg. Doctoral dissertation.

Orwa C, A Mutua, Kindt R , Jamnadass R, S Anthony. 2009 Agroforestree Database:a tree reference and selection guide version 4.0. Available at: (http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp) (Accessed 21 November 2014)

Pacific bulb society. 2014. Online. Eucomis Tree. Available at: http://www.pacificbulbsociety.org/pbswiki/index.php/EucomisThree (Accessed 18 November 2014).

Palmer, E., Pitman, N., 1972. Trees of Southern Africa. Balkema publishers, Cape Town

Petal faire nursery. 2014. Online. Plants: *Cassinopsis ilicifolia*. Available at: http://www.petalfaire.co.za/index.php?content=plants&id=571&view=all (Accessed on 12 November 2014).

Philcox, D., 1990. Scrophulariaceae. In: Launert, E. & Pope, G.V. (editors). Flora Zambesiaca 8 (2). Flora Zembesiaca maganing Committee, London, United Kingdom, pg 179.

Pignatti, S. 1982. Flora d'Italia. Edagricole, Bologna, Italy (in Italian).

Plant delight nursery,Inc. 2014. *Eucomis autumnalis* ssp. *Clavata.* Available at: http://www.plantdelights.com/Eucomis-autumnalis-ssp-clavata-for-sale/Buy-Autumn-Pineapple-Lily/ (Accessed 30 November 2014).

© University of Pretoria

Plant lust. 2014.Online. *Eucomis autumnalis* ssp. *Clavata.*Available at: http://plantlust.com/plants/eucomis-autumnalis-ssp-clavata/ (Accessed 30 November 2014).

Plants for a future. 2014. Online. *Typha minima*. Available at: http://www.pfaf.org/user/Plant.aspx?LatinName=Typha+minima (Accessed on 21 November 2014).

Plants photographed on Kyffhauser. 2014. Online. *Withania somnifera* (L.) Dunal. Available at: http://www.kyffhauser.co.za/Plants1/Withania_somnifera/Image4.htm (Accessed on 21 November 2014).

Plantzafrica. 2003. Online. *Dracaena aletriformis* (Haw.) Bos. Available at: http://www.plantzafrica.com/plantcd/dracaenalet.htm (Accessed 15 November 2014).

Plantzafrica. 2009. Online. *Aloe plicatilis* (L.) Mill. Available at: http://www.plantzafrica.com/plantab/aloeplicatilis.htm (Accessed on 12 November 2014).

Plantzafrica. 2009. Online. *Ficus sur* Forssk.. Available at: http://www.plantzafrica.com/plantefg/ficussur.htm (Accessed on 20 November 2014).

Plantzafrica. 2014. Online. *Eucomis vadermerwei* I.Verd. Available at: http://www.plantzafrica.com/plantefg/eucomisvandermerwei.htm (Accessed 30 October 2014).

Plantzafrica. 2014. Online. *Euphorbia tirucalli* L. Available at: http://www.plantzafrica.com/plantefg/euphorbtirucal.htm (Accessed on 30 November 2014).

Plantzafrica. 2014. Online. *Leonotis leonurus* (L.) R.Br. Available at: http://www.plantzafrica.com/plantklm/leonotisleon.htm. (Accessed 25 November 2014).

Plantzafrica. 2014. Online. *Merwilla plumbea* (Lindl.) Speta. Available at: http://www.plantzafrica.com/plantklm/merwilplum.htm (Accessed 29 November 2014).

Plantzafrica. 2014. Online. *Tarchonanthus camphoratus* L. Available at: http://www.plantzafrica.com/planttuv/tarchoncamphor.htm (Accessed 18 November 2014).

Plantzafrica. 2014. Online. *Typha capensis* (Rohrb.) N.E.Br. Available at: http://www.plantzafrica.com/planttuv/typhacapen.htm (Accessed on 27 November 2014).

Plantzafrica.com. 2009. Online. *Faurea saligna*. Available at: http://www.plantzafrica.com/plantefg/faureasal.htm (Accessed 25 July 2014).

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Plantzafrica.com. 2014. Online. *Salvia africana lutea* L. Available at: http://www.plantzafrica.com/plantqrs/salviaafricanlut.htm (Accessed on 28 November 2014).

Plantzafrica.com. 2014. Online. *Withania somnifera* (L.) Dunal Available at: http://www.plantzafrica.com/plantwxyz/withaniasomnifera.htm (Accessed on 21 November 2014).

Pooley, E., 2003. Mountain Flowers. A guide to the flora of the Drakensberg and Lesotho. The flora publications trust, Durban.

Prota4u. 2006. Online. *Ficus sycomorus* L.. Available at: http://www.prota4u.org/protav8.asp?p=Ficus+sycomorus (Accessed on 21 November 2014).

Rousseae, F.,1970. Proteaceae of Southern Africa. Purnell, Cape Town, Johannesburg, London.

The IUCN red list threatened species. 2014. Online. *Dracaena draco.* Available at: http://www.iucnredlist.org/details/30394/1 (Accessed 15 November 2014).

Till-Bottraud, I., Poncet, B. N., Rioux, D., Girel, J., 2010. Spatial structure and clonal distribution of genotypes in the rare Typha minima Hoppe (Typhaceae) along a river system.Botanica Helvetica 120(1), 53-62.

Van Wyk, B. E., 2008. A review of Khoi-San and Cape Dutch medical ethnobotany. Journal of Ethnopharmacology 119(3), 331-341.

Van Wyk, B. E., Gericke, N., 2000. People's plants: A guide to useful plants of Southern Africa. Briza Publications.

Van Wyk, B. E., Oudtshoorn, B. V., Gericke, N., 1997. Medicinal Plants of South Africa. Briza.

Van Wyk, B., Malan,S., 1998. Field guide to the wild flowers of the Highveld. Struik publishers (Pty) Ltd.

Van Wyk, P., 1984. Fied guide to the trees of Kruger National Park. Struik publishers (Pty) Ltd, Cape Town.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The medicinal and poisonous plants of Southern and Eastern Africa. E & S Livingstone LTD. Edinburgh and London.

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Chapter 3

Antimycobacterial activity of plant extracts against *Mycobacterium tuberculosis* and Cytotoxicity in U937 cell line

Twenty South African medicinal plant extracts were evaluated for *in vitro* antimycobacterial activity using the Microtitre Alamar Blue Assay and Cytotoxicity with XTT assay. *Mycobacterium tuberculosis* H37Rv strain was used as the test organism. Cytotoxicity test was done on human macrophages (U937 cell line). Of all the 20 extracts 7 showed activity against *Mycobacterium* with a minimum inhibitory concentration ranging from 125-31.25 µg/ml. *Ficus sur* had a selectivity index of 3.

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3. Background

Based on earlier research, the rich flora of South Africa has indicated great potential against multiple bacteria. *In vitro* Work has been done against *Mycobacterium tuberculosis* using many medicinal plants (McGaw *et al*., 2008; Mativandlela *et al*., 2008; Green *et al*., 2010). The antimycobacterial activity of the selected South African medicinal plants extracts was determined using a Microtitre Alamar Blue Assay (MABA).

3.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis an actinobacteria was first discovered by German physician and scientist, Robert Koch in 1882 (Kaufmann, 2000). The bacterium is the causative agent of the second leading infectious disease worldwide Tuberculosis (TB) (WHO, 2014). Tuberculosis is a respiratory infection frequently affecting the lungs (McGaw *et al*., 2008).

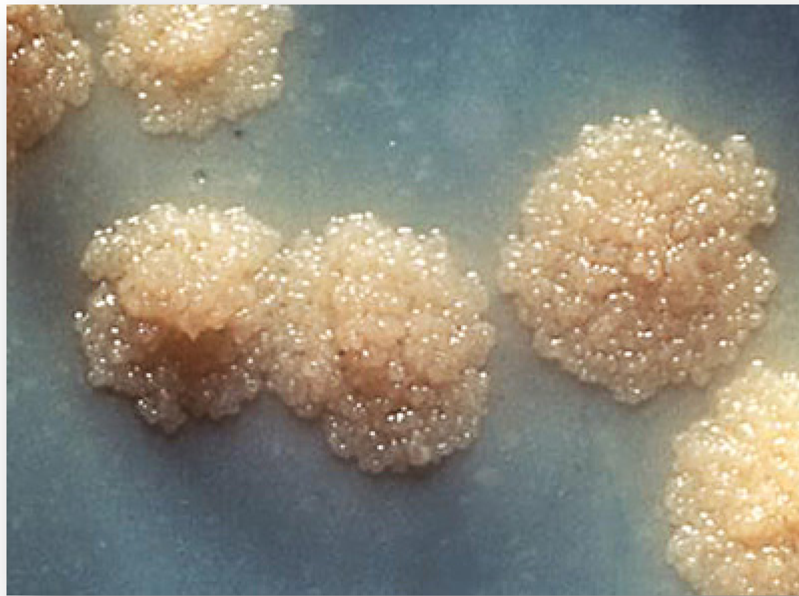

Figure 3.1.1: Microscopic view of *Mycobacterium tuberculosis* (www.asbmb.org, 2014)

The bacilli is characterised by its slow growing, gram-positive, rod-shaped and lipid rich cell wall (Figure 3.1.1) (Cole *et al*., 2002). It is known as one of the emerging infectious diseases (Morens & Fauci, 2012). This gram-positive obligate aerobe bacterium can be distinguished by permeability bilayer barrier; composed of mycolic acids. (WebMD, 2005, Gautam *et al*., 2004).

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3.1.1 Materials & Methods

3.1.1.1 Material: *Mycobacterium tuberculosis* H37Rv strain

Mycobacterium tuberculosis H37Rv strain (ATCC 27264) was kindly sponsored by the National Health Laboratories at the University of Pretoria. The bacterium was cultured onto Middlebrook 7H11 agar base (7H11) and grown on the media for 2 weeks at 37ºC at the University of Pretoria Medical Campus in a Biohazard 3 facility.

3.1.1.2 Inoculum and drug preparation (Positive controls)

After 2 weeks of growth on Middlebrook 7H11 agar base, suspensions were prepared in 7H9 broth media so that their turbidity matched that of a McFarland no.1 turbidity standard. The suspensions were further diluted 1:25 in 7H9 broth base. Isoniazid (INH) and rifampicin (RMP) were obtained from Sigma Aldrich. Stock solution of INH was prepared in deionized water and RMP was prepared in dimethyl sulfoxide (DMSO). Stock solutions were diluted in 7H9 broth to two times the maximum desired final testing concentration prior to their addition to microplates (Franzblau *et al*., 1998).

3.1.2 Microtitre Alamar Blue Assay (MABA) used to determine the antimycobacterial activity of plant extracts on *Mycobacterium tuberculosis*

The Microtitre Alamar Blue Assay is used to determine the viability of cells, it has been used as a nonradioactive assay to monitor and quantify lymphocyte proliferation and also fungi and bacteria. The assay is based on the reduction of Alamar blue dye which is used as general indicator of cellular growth (Collins & Franzblau, 1997; McBride *et al*., 2005).

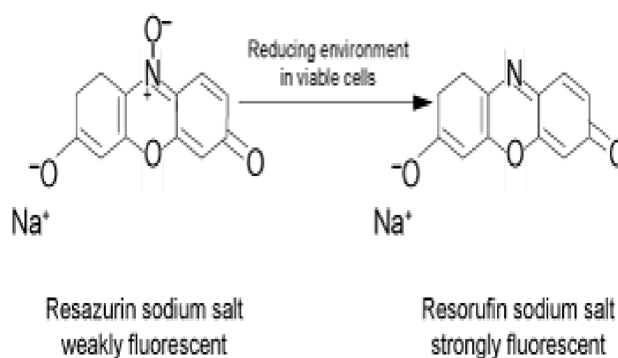

Figure 3.1.2: An illustration of the reduction of Resazurin to Resorufin sodium salt in viable cells (www.bmglabtech.com, 2014)

Alamar blue dye is a resazurin indicator. It is a blue non-flourescent and non-toxic dye (Sarker *et al*., 2007). This assay measures innate cellular metabolic activity which reduces

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Alamar blue dye and changes its colour to pink (fluorescent) (Figure 3.1.2) (O`Brien *et al*., 2000). When reduced to resofurin by oxidoreductases this measures the amount of viable cells that are present in the test sample. Specifically Alamar blue is reduced by NAPDH, reduced flavin adenine dinucleotide, reduced flavin mononucleotide and the cytochromes produced inside the cells (Collins & Franzblau, 1997; McBride *et al*., 2005).

Most assays used to screen antimycobacterial activity against *M. tuberculosis* lack a few desired attributes for large scale screening, and some are expensive too. Microtitre Alamar Blue Assay was first described in 1997 by Scott G. Franzblau. The assay has certain advantages as compared to other assays in that it is rapid, simple, inexpensive, high-throughput and can screen compounds against any isolate, not only recombinant strains. The main advantage of using MABA is that growth can be evaluated: fluorometrically, spectrophotometrically and visually and it can be done without the use of specialized equipment. Laboratories with limited resources can also use MABA (Collins & Franzblau, 1997).

3.1.2.1 Experimental procedure (MABA)

Two hundred microliters of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. The wells in rows B to G in columns 3 to 11 received 100 ml of 7H9 broth (Sigma Aldrich), supplemented with 10% OADC and 2% PANTA. One hundred microliters of 20 plant extracts were added to the wells in rows B to G in columns 2 and 3. By using a multichannel pipette, 100ml was transferred from column 3 to column 4, and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued through column 10, and 100 ml of excess medium was discarded from the wells in column 10. Final drug concentration ranges were as follows: for INH, 0.031 to 8.0 mg/ml; for RMP, 0.0156 to 4 mg/ml. One hundred microliters of *M. tuberculosis* inoculum was added to the wells in rows B to G in columns 2 to 11 by using an Eppendorf repeating pipette (yielding a final volume of 200 ml per well). Thus, the wells in column 11 served as drug-free (inoculum-only) controls. The plates were sealed with Parafilm and were incubated at 37°C for 5 days. Fifty microliters of a freshly prepared 1:1 mixture of 103 Alamar Blue reagent and 10% Tween 80 was added to well B11. The plates were reincubated at 37°C for 24 h. If well B11 turned pink, the reagent mixture was added to all wells in the microplate (if the well remained blue, the reagent mixture would be added to another control well and the result would be read on the

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following day). The microplates were resealed with parafilm and were incubated for an additional 24 h at 37°C, and the colours of all wells were recorded. A blue colour in the well was interpreted as no growth, and a pink colour was scored as growth. A few wells appeared violet after 24 h of incubation, but they invariably changed to pink after another day of incubation and thus were scored as growth (while the adjacent blue wells remained blue). The MIC was defined as the lowest drug concentration which prevented a colour change from blue to pink (Franzblau *et al*., 1998).

3.1.3 Results & Discussion

All the 20 ethanol plant extracts were screened for antimycobacterial activity at least at three independent experiments in replicates of three for each experiment. Out of the 20 tested plant extracts, 7 showed the best activity when compared to the rest of the extracts against *M. tuberculosis* (Table 3.1).

Table 3.1: Minimal inhibitory concentrations (MIC in µg/ml) of plant extracts against *M. tuberculosis*

Plant extract	MIC (µg/ml)
Alectra sessiliflora	**125**
Aloe plicatilis	1000
Cassinopsis ilicifolia	500
Dracaena aletriformis	500
Dracaena draco	500
Eucomis autumnalis ssp. clavata	1000
Eucomis humilis	1000
Eucomis vandermerwei	1000
Euphorbia tirucalli	**125**
Faurea saligna	>1000
Ficus sur	**62.5**
Ficus sycomorus	1000
Leonotis leonurus	**125**
Merwillia plumbea	500
Salvia african- lutea	**<31.25**
Sphedamnocarpus pruriens	**62.5**
Tarchonanthus camphorates	500
Typha capensis	1000
Typha minima	500
Withania somnifera	**125**
Isoniazid	<0.156
Rifampicin	<0.156

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The positive controls, Isoniazid and Rifampicin both showed a minimum inhibitory concentration (MIC) of less than <0.156µg/ml as reported in an article by Adaikkappan *et al*., 2012. The plant extracts that were most effective against *M.tuberculosis* H37Rv strain were *Ficus sur* and *Sphedamnocarpus pruriens* both with an MIC of 62.5µg/ml. This was followed by *Alectra sessiliflora, Euphorbia tirucalli*, *Leonotis leonurus L.*and *Withania somnifera* they showed MICs of 125µg/ml. *Salvia africana-lutea* exhibited an MIC of <31.25µg/ml (Table 3.1).

It can be speculated that the above mentioned plants have specific plant chemical constituents that target certain elements of *Mycobacterium* and interfere with bacterial growth, leading to moderate inhibition of growth. In a recent study by Madikizela *et al*., 2014 *Ficus sur* traditionally used to treat pulmonary tuberculosis (Eldeen *et al*., 2005; Lawal *et al*., 2014; McGaw *et al*., 2008) showed antimycobacterial activity with an MIC of 6.25mg/ml (6250µg/ml) which is 100 folds less concentration as compared to the present study (Eldeen *et al*., 2005; Lawal *et al*., 2014; McGaw *et al*., 2008). It was also observed that the ethanol leaf extract of *F. sur* showed an MIC of 3.12mg/ml (3120µg/ml) (Eldeen & Van Staden., 2007). The ecological environment of plants differs among the reported and present study, which could result in biochemical differences in the *F.sur* plant. The plant part used in Madikizela *et al*., 2014 was the bulb whereas the ethanol leaf extract was used in this study. According to literature no antimycobacterial activity has been reported on *S.pruriens*.

Alectra sessiliflora is one of the medicinal plants used for tuberculosis management in Nigeria (Ogbole & Ajaiyeoba, 2009). The MIC of this plant in the present study was found to be 125µg/ml. This is the first record of antimycobacterial activity of *A. sessiliflora*. The plant exhibited antibacterial activity in previous studies against *Staphylococcus aureus*, *Psedomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae* and *Bacillus pumilus* at an MIC of 3.13-25.0mg/ml.

In a study by Marrita *et al*., 2010 antimycobacterial activity of *E. tirucalli* was reported at an MIC of 2mg/ml using the BACTEC MGIT 960 system. This could be due to its high tannin, flavonoids and terpenoids content (Marrita *et al*., 2010). The presence of diterpenoids and triterpenoids in Euphorbiaceae family plants (de Oliveira *et al*., 2014) could be responsible for their *in vivo* and *in vitro* activity, which could be responsible for the antimycobacterial activity in the present study. The difference in method used for determining the

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antimycobacterial activity could be reason for difference in MIC. The difference in the source of the plant material could also be the reason for the contrast in the results.

Salvia Africana lutea from the Lamiaceae family showed activity at an MIC of <31.25µg/ml in the present study. Antimycobacterial efficiency of methanol: chloroform (1:1) extract of *Salvia African-lutea* was determined by Kamatou *et al*., 2007. It showed an MIC of 0.50mg/ml (500µg/ml). The aerial parts acetone extract showed activity against *Mycobacterium tuberculosis* at a concentration of 312.50µg/ml (Nielsen *et al*., 2012). It has been reported by Seaman 2005 that *S.africana lutea* methanol plant extract has antimycobacterial activity with an MIC of 1mg/ml (1000µg/ml) this result was obtained using the BACTEC 460 method. In a paper by Hussein *et al*., 2007 Four diterpenoid compounds namely compounds 2, 3, 4 and 6 (Figure 3.1.3) were evaluated for antimycobacterial activity. Compounds 3 and 6 isolated from *S.african lutea* (Figure 3.1.3) showed activity of 157 and 28µM respectively. The present study showed a better result of antimycobacterial activity as compared to the reported results, which could be due to the ethanol solvent being used for extraction and probably high concentration of diterponoids as significant activity was observed by Hussein et al,. 2007.

Another plant from the Lamiaceae family; *Leonotis leonurus* L. is traditionally used for coughs, influenza and respiratory ailments (Makunga *et al*., 2008; McGaw *et al*., 2008). Biochemical analysis of the plant extract indicated potential bioactive compounds known as diterpernoids (Naidoo *et al*., 2011), which could contribute to the antimycobacterial activity observed in this study.

Withania somnifera L. Dunal (Solanaceae) aqueous extract showed antimycobacterial activity exhibiting an MIC of 1.0mg/ml (1000µg/ml) (Adaikkappan *et al*., 2012, Gautam *et al*., 2004). The ethanol extract in this study showed an MIC of 125µg/ml, which is significantly lower as compared to reported MIC. The difference in the use of solvents, could possibly extrapolate different compounds based on the polarity can be the reason for the contrast in activity.

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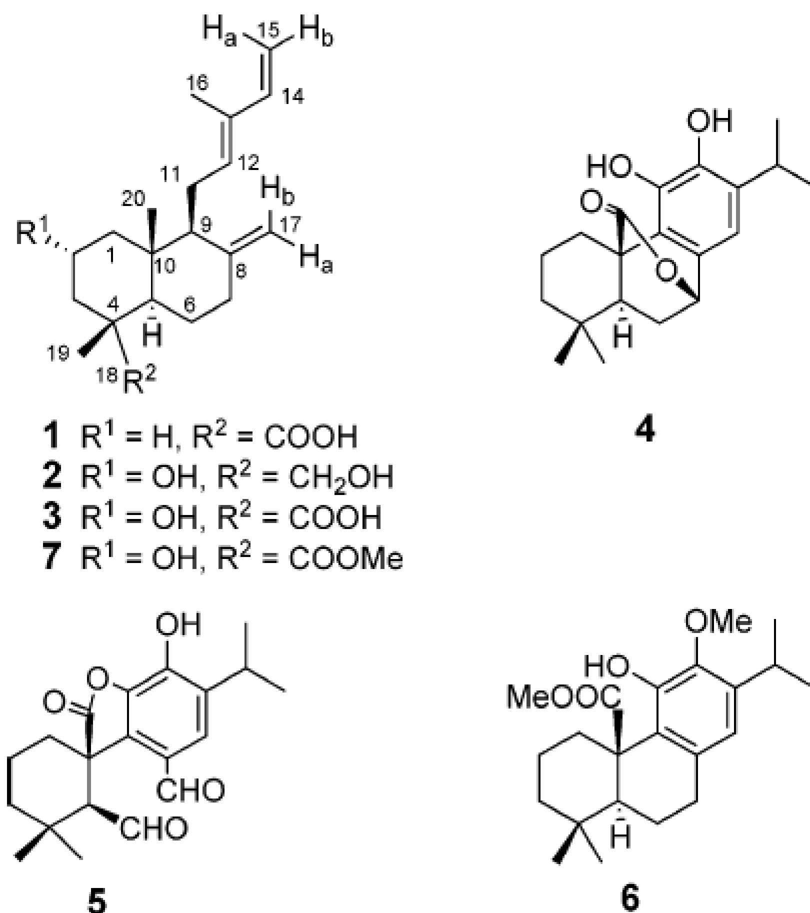

Figure 3.1.3: Diterpenoid compounds isolated from *Salvia africana-lutea* (Hussein *et al.*, 2007)

In a study conducted by Mativandlela *et al.*, 2008, seven plants were selected based on their traditional use to treat TB related symptoms; these were tested against *M. tuberculosis.* Three of the plant extracts namely, *Galenia africana L., Artemisia afra* Jacq. Ex Willd and *Dodonaea angustifolia* L. f. showed antimycobacterial activity against *M. tuberculosis* at MICs of 780µg/ml, 1560µg/ml and 3130µg/ml respectively. In a paper by Newton *et al.*, 2002, forty-three plant species were selected based on their traditional use to treat TB or leprosy. These plants were screened for antimycobacterial activity against *M. aurum* and *M. smegmatis*. Crude methanolic extracts from the plants namely *Commiphora mukul, Psoralea corylifolia, Sanguinaria canadensis* had significant antimycobacterial activity against *M. aurum* only with an MIC of 62.5µg/ml. When comparing the MICs of the tested plants to those reported in Mativandlela *et al.*, 2008 *S. africana lutea* and *S. pruriens* were more effective in combating *M. tuberculosis* than the above mentioned plant extracts.

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3.1.4 Conclusion

The ethanol plant extracts were evaluated for antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv strain using the MABA method. *Leonotis leonurus* (125µg/ml), *Withania somnifera* (125µg/ml) and *Salvia africana lutea* (<31.25µg/ml) had significant activity with the latter being more significant. This could be due to the bioactive constituents. *S. Africana lutea* contains diterpenoids which have been isolated and reported for antimycobacterial activity (Hussein *et al*., 2007). *Leonotis leonurus* biochemical analysis showed presence of diterpenoids. Therefore the diterpenoids could have potentially bioactive against *Mycobacterium tuberculosis*. *Ficus sur* and *Sphedamnocarpus pruriens* (62.5µg/ml) both showed significant antimycobacterial activity against *Mycobacterium tuberculosis.*

3.2 Cytotoxicity

Cells can be defined as the structural, functional and biological units of all organisms (biology-online.org). They are a critical part of human development. For this reason substances exposed to living organism need to be evaluated for their level of toxicity to cells, this process is known as Cytotoxicity. Toxicity analysis is used to determine how toxic a substance is to cells and if is safe for human consumption. The selected medicinal plant extracts were tested for cytotoxicity against the U937 cell line. The aim was to determine 50% inhibitory concentration (IC_{50}) on U937 cell line.

Human macrophage (U937) cell line a circular structure with a single well-defined nucleus (Figure 3.2.1). This cell line has immature cells which can be differentiated to mature monocytes (macrophages) or dendritic cells (Sharp, 2013). Sundstrom and Nilsson in 1974 derived U937 cell line from malignant cells of a Caucasian 37 year old male patient with histiocytic lymphoma (ATCC, 2008).Macrophages are the first line of defence against pathogenic organisms, phagocytic white blood cells (Danelishvili *et al*., 2003).

The U937 cell line was selected to be screened for cytotoxicity due to the fact that *Mycobacterium tuberculosis* is found in alveolar macrophages within living organisms (Danelishvili *et al*., 2003). The cell line was used to determine the toxicity of the crude plant extracts using a tetrazolium colometric assay which involves the incubation of viable macrophages test model, with the test sample. The results were read using Spectrophotometer (ELISA reader). Advantages associated with this are that it is rapid, inexpensive, and precise (Mosmann, 1983, Ferrari *et al*., 1990, Van de Loosdrecht *et al*., 1994).

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U937 cell line

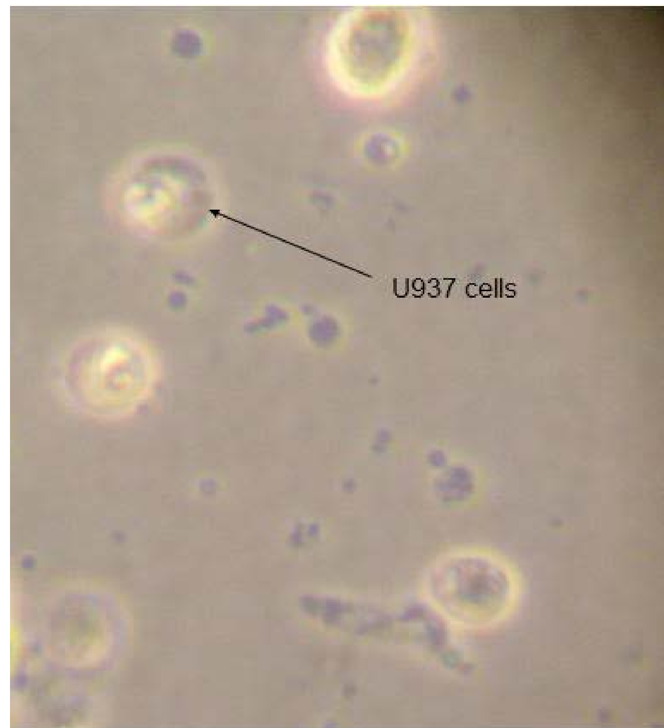

Figure 3.2.1: Microscopic representation of U937 cells after thawing (protocol-online.org, 2005).

3.2.1 Materials & Methods

3.2.1.1 Culturing of U937 cells

The cytotoxicity assays were conducted by Ms. Mabatho Nqephe at the University of Pretoria

Undifferentiated U937 cells (obtained from Sigma-Aldrich 2012, South Africa) were maintained in 10% Roswell Park Memorial Institute (RPMI) 1640 medium (Highveld biological, South Africa) which was supplemented with 10% heat activated foetal bovine serum (FBS), 2mM L-glutamine and 0.1% antimicrobial solution (penicillin, streptomycin and fungizone) at a pH of 7.2. The cells were cultured every 2-3 days after a confluent monolayer had formed.

3.2.1.2 Counting of cells with a hemocytometer

Hemocytometer was used to count the number of cells in order to establish the correct concentration for cytotoxicity assay. During the sub-culturing cells that had attached to the culture flask, were trypsinized (0.25% trypsin, containing 0.01% EDTA) for ten minutes at 37^{0}C and stopped by the addition of complete medium. The cells with complete medium were centrifuged for 5 minutes at 980rpm to form a pellet. Hemocytometer was used to count

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the number of cells in order to establish the correct concentration for cytotoxicity assay. The cells were re-suspended in complete medium (5%FBS + MEM + PS) to give a cloudy suspension (2-4 ml). In an eppendorf tube a cell suspension of 1:10 dilutions was prepared in trypan blue solution (10µl cells in 90µl trypan blue). A volume of 10µl of cell suspension was added to the hemocytometer (Figure 3.2.2). The cells were counted using a hand-held tally under a microscope. The concentration of cells was determined using the following calculation:

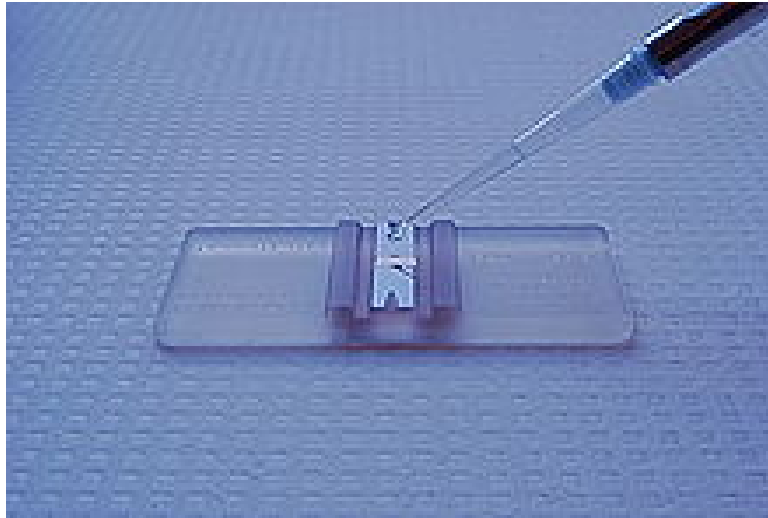

Figure 3.2.2: Hemocytometer used to count the number of cells (Abcam.com, 2012)

cells counted per square = # cells counted/4

Cell concentration (cell suspension) = # cells counted per square X 10 X 10000

= cells per ml

Volume added to cell pellet = Cell concentration wanted X volume wanted / Concentration of cells in suspension

= Total volume

Volume wanted = Total volume – volume added to cell pellet

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3.2.1.3 XTT Cytotoxicity Assay

A cell proliferation assay kit is used to assess cell cycle regulatory factors such as cytokines, mitogens and drugs. It is an easy to use tool for understanding the induction and proliferation of cells in *in vitro* models. The principle of the assay is based on the extracellular reduction of XTT to a water soluble formazan which dissolves directly in culture medium; this reaction is catalysed by NADH (Francoeur & Assalian, 1996; Berridge *et al*., 2005). In order to analyse the number of viable cells the tetrazolium salt needs to be cleaved to an orange formazan dye by the principles mentioned (Figure 3.2.3) (Roche, 2005).

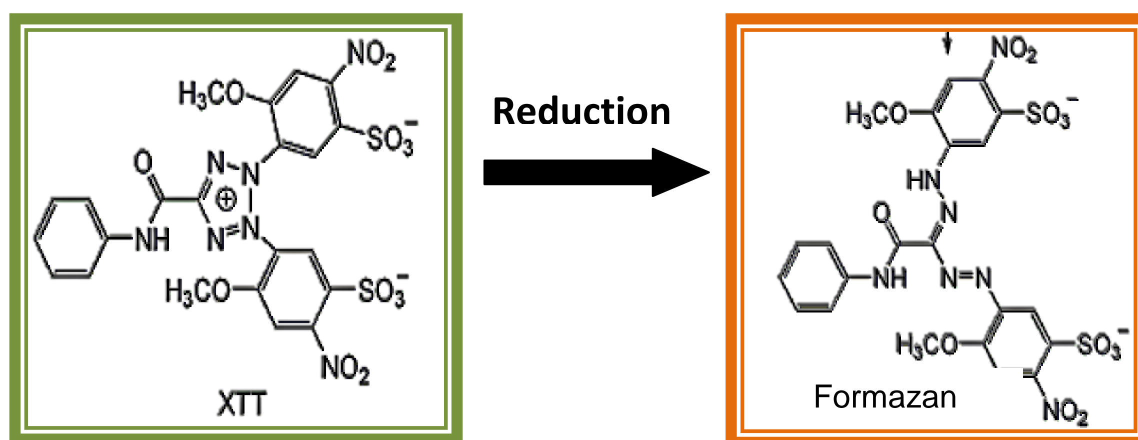

Figure 3.2.3: Reduction reaction of XTT to formazan (biosynth.com, 2012)

Cytotoxicity test

The cytotoxicity of crude extract from the twenty plant extracts was investigated by using XTT-based colorimetric assay Cell Proliferation Kit II (Boehringer-Mannheim) following the method as described by Roche (2005). The extracts were dissolved in 100µl of dimethyl sulfoxide (DMSO). The crude plant extracts were serially diluted from the highest tested concentration (400 µg/ml) to the lowest tested concentration of 3.125µg/ml. Inhibitory concentration at which 50% (IC50) of the U937 cells were viable until the 4th day was considered to be the highest concentration which is non-toxic to the cells. These values were calculated using Graph Pad Prism 4 programme (Mathabe *et al*., 2008).

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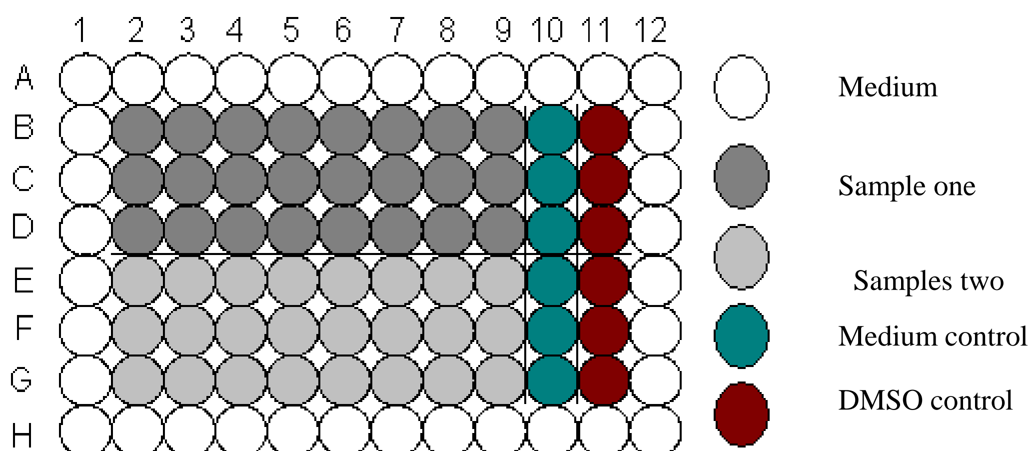
Sample plate



Reference plate

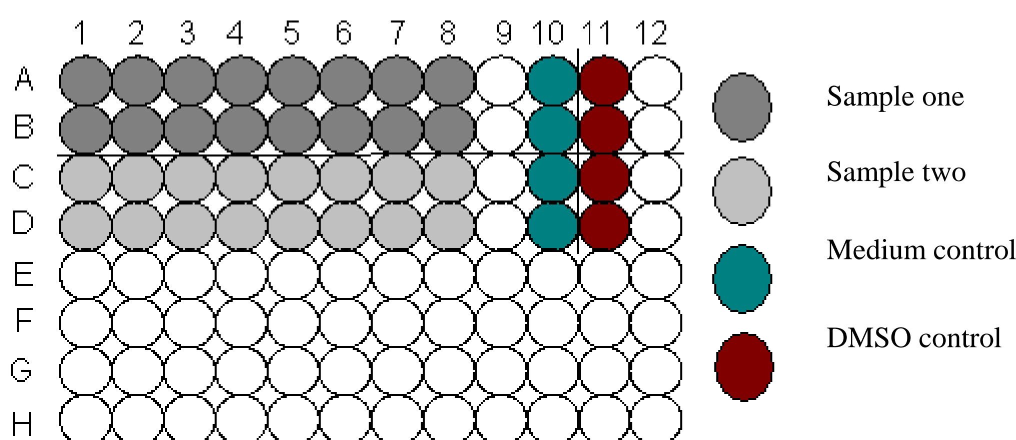


Figure 3.2.4: Layout of 96-well plates used to investigate Cytotoxicity of plant extracts

3.2.2 Results & Discussion

The level of toxicity of the 20 plant extracts was determined using the XTT Cytotoxicity assay. The plant extracts showed moderate toxicity to the human macrophages when compared to the Actinomycin D (positive control) (Table 3.2). A benchmark of 50µg/ml was used to indicate 50% inhibitory activity of the plant extracts in other words a plant extract with an IC_{50} of less than 50µg/ml was considered toxic. Some of the plant extracts were toxic to the U937 cell line.

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Table 3.2: Cytotoxic evaluation of ethanol plant extracts on U937 cell line

Plant extract	IC_{50}(µg/ml)
Alectra sessiliflora	110.2
Aloe plicatilis	**38.31**
Cassinopsis ilicifolia	141
Dracaena aletriformis	90.74
Dracaena draco	**20.82**
Eucomis autumnalis ssp. Clavata	68.50
Eucomis humilis	57.29
Eucomis vandermerwei	51.44
Euphorbia tirrucalli	14.76
Faurea saligna	202.4
Ficus sur	210.2
Ficus sycomorus	**30.38**
Leonotis leonurus	39.98
Merwillia plumbea	**22.49**
Salvia african- lutea	83.85
Sphedamnocarpus pruriens	125.9
Tarchonanthus camphorates	**25.23**
Typha capensis	**24.47**
Typha minima	198.2
Withania somnifera	**6.810**
Actinomycin D	0.00932

Withania somnifera indicated significant toxicity to the U937 cell line. This toxicity can be a result of the new steroid compound; 5, 6-De-epoxy-5-en-7-one-17-hydroxy withaferin A, which was isolated from aerial parts of *W. Somnifera*. The compound withaferin A was found to be very toxic when screened against a variety of cancer cell lines, MCF-7 breast & WRL-68 liver lines with IC_{50} of 1.0µg/ml. Moderate toxicity was observed on prostate (PC-3) and Colon (CACO-2) cell lines with an IC50 of 7.4µg/ml and 3.4µg/ml respectively (Siddique *et al*., 2014). *Euphorbia tirrucali* has been reported to be toxic due to the latex it contains (Silva *et al*., 2007).

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To further determine the efficacy of the plant extracts against *M .tuberculosis*, selective index (SI) of each sample was calculated (Table 3.3). In the present study SI was determined using equation(1).

$$\mathrm{SI} = \mathrm{IC50} / \mathrm{MIC} \qquad (1)$$

Table 3.3: Selectivity index of plant extracts on human macrophages and bacterial cells

Plant extract	IC_{50}(µg/ml)	MIC(µg/ml)	SI
Alectra sessiliflora	110.2	125	0.882
Aloe plicatilis	38.31	1000	0.038
Cassinopsis ilicifolia	141	500	0.282
Dracaena aletriformis	90.74	500	0.181
Dracaena draco	20.82	500	0.042
Eucomis autumnalis ssp. clavata	68.50	1000	0.069
Eucomis humilis	57.29	1000	0.057
Eucomis vandermerwei	51.44	1000	0.051
Euphorbia tirrucalli	14.76	125	0.118
Faurea saligna	202.4	>1000	0.202
Ficus sur	**210.2**	**62.5**	**3.363**
Ficus sycomorus	30.38	1000	0.030
Leonotis leonurus	39.98	125	0.320
Merwillia plumbea	22.49	500	0.045
Salvia african- lutea	**83.85**	**<31.25**	**2.683**
Sphedamnocarpus pruriens	**125.9**	**62.5**	**2.014**
Tarchonanthus camphorates	25.23	500	0.050
Typha capensis	24.47	1000	0.024
Typha minima	198.2	500	0.396
Withania somnifera	6.810	125	0.054

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Selectivity index was used to distinguish toxicity that is due to plant extracts and toxicity in general (Adamu *et al*., 2014; de Prince *et al*., 2012). *Ficus sur* showed the highest selectivity index of 3.363 (Table 3.3) in this study; which means it is three times more effective against *M .tuberculosis* as compared to Human macrophages (de Prince *et al*., 2012). Plant extracts with high selectivity indexes and low or moderate toxicity; can be considered as good candidates for inclusion in drug development.

3.2.3 Conclusion

Twenty South African medicinal plant extracts were evaluated for antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv strain. Of all the 20 extracts seven showed good activity. The extracts were further probed for cytotoxicity in human macrophages (U937). Most of the extracts showed moderate toxicity. *Withania somnifera* seemed very toxic towards the macrophages; this could be due to the phytochemical composition. Selectivity index was done to determine the efficacy of the plant extracts against bacterial cells as compared to the macrophages. *Ficus sur* had the highest selectivity index followed by *S. Africana-lutea* and *S. pruriens*. These three plant extracts are good candidates for further studies.

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3.4 References

Abcam. com. 2012. Online. U937 (Human leukemic monocyte lymphoma cell line) cytoplasmic lysate (ab14904). Available at: http://www.abcam.com/U937-Human-leukemic-monocyte-lymphoma-cell-line-Cytoplasmic-Lysate-ab14904.html (Accessed 07 December 2014).

Adaikkappan, P., Kannapiran, M., Anthonisamy, A., 2012. Anti-mycobacterial activity of *Withania somnifera* and *Pueraria tuberosa* against *Mycobacterium tuberculosis* H37Rv.Journal of Academic Industrial Resolution 1(4), 153-156.

Adamu, M., Naidoo, V., Eloff, J. N., 2014. The antibacterial activity, antioxidant activity and selectivity index of leaf extracts of thirteen South African tree species used in ethnoveterinary medicine to treat helminth infections. BMC veterinary research 10(1), 52.

ATCC – The global bioresource center. 2008. Product description. Online. Available:http://www.tcc.org/ATCCAdvance CatalogSearch/ProductDetails (Accessed 06 November 2014).

Berridge, M.V., Tan, A.S., Herst, P.M., 2005. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnology Annual Review 11 127-152.

Biology online. 2008. Online. Cell definition. Avaible at: http://www.biology-online.org/dictionary/Cell (Accessed on 30 September 2014).

Biosynth chemistry and bilogy. 2012. Dyes, stains, labels and indicators. Available at: http://www.biosynth.com/index.asp?topic_id=214&g=19&m=285 (Accessed 07 December 2014).

BMG labtech. 2014. Online. Alamar blue assay for assessment of cell proliferation using the FLUOstar OPTIMA. Available at: http://www.bmglabtech.com/en/applications/application-notes/195-alamarblue-assay-for-assessment-of-cell-proliferation-using-the-fluostar-optima-obj-91-804.html (Accessed 30 September 2014).

Cole, E. T., Scott, R. A., Connor, A. L., Wilding, I. R., Petereit, H. U., Schminke, C., Cadé, D.,2002. Enteric coated HPMC capsules designed to achieve intestinal targeting. International Journal of Pharmaceutics 231(1), 83-95.

Collins, L., Franzblau, S.G., 1997. Microplate Alamar blue assay verusus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrobial agents and Chemotherapy 41(5), 1004-1009.

Danelishvili, L., McGarvey, J., Li, Y. J.,Bermudez, L. E., 2003. *Mycobacterium tuberculosis* infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. Cellular microbiology, 5(9), 649-660.

© University of Pretoria

de Oliveira, L.F.S., Fuentefria, A.M., da Silva Klein, F., Machado, M.M., 2014. Antifungal activity against *Cryptococcus neoformas* strains and genotoxicity assessment in human leukocyte cells of *Euphorbia tirucalli* L.. Brazilian Journal of Microbiology 45(4), 1349-1355.

de Prince, K.a., sordid, R., Pavan, F.R., Santos, A.C.B., Araujo, A.R., Leite, S.R.A., Leite, C.Q.F., 2012. Anti-*Mycobacterium tuberculosis* activity of fungus *Phomopsis stipata*. Brazilian Journal of Microbioilogy 2012, 224-229.

Eldeen, I. M. S., Van Staden, J., 2007. Antimycobacterial activity of some trees used in South African traditional medicine. South African Journal of Botany 73(2), 248-251.

Eldeen, I.M.S., Elgorashi, E.E., van Staden, J., 2005. Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. Journal of Ethnopharmacology 102, 457-464.

Ferrari, M., Fornasiero, M. C., Isetta, A. M.,1990. MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. Journal of immunological methods, 131(2), 165-172.

Francoeur, A., and Assalian, A., 1996. Microcat: A novel cell proliferation and cytotoxicity assay based on WST-1. Biochemica 3 19-25.

Franzblau,S.G., Witzig,R.s., McLaughlin, J.C., Torres, P., Madico,G., Hernandez,A., Degnan, M.T., Cook, M.B., Quenzer,V.K., Ferguson,R.M., Gilman, R.H., 1998. Rapid, Low-Technology MIC determination withclinical Mycobacterium tuberculosis isolates by using the Microplate Alamar Blue assay. Journal of Clinical Microbiology 36(2), 362-366.

Gautam, M., Diwanay, S. S., Gairola, S., Shinde, Y. S., Jadhav, S. S., Patwardhan, B. K., 2004. Immune response modulation to DPT vaccine by aqueous extract of *Withania somnifera* in experimental system. International immunopharmacology 4(6), 841-849.

Green, E., Samie, A., Obi, C. L., Bessong, P. O., Ndip, R. N., 2010. Inhibitory properties of selected South African medicinal plants against *Mycobacterium tuberculosis*. Journal of ethnopharmacology 130(1), 151-157.

Hussein, A.A., Meyer, J.J.M., Jimeno, M.L., Rodriguez, B., 2007. Bioactive diterpenes from *Orthosiphon labiatus* and *Salvia africana-lutea*. Journal of Natural Products 70, 293-295.

Kamatou, G.P.P., Van Vuuren, S.F., Van Heerden, F.R., Seaman, T., Viljoen, A.M., 2007. Antibacterial and antimycobacterial activities of South African Salvia species and I solated compounds from *S. chamelaeagnea*. South African Journal of Botany 73, 552-557.

Kaufmann, S.H.E., 2000. Is the development of a new tuberculosis vaccine possible? Nature Medicine 6, 955-960.

© University of Pretoria

Lawal, I.O., Grierson, D.S., Afolayan, A.J., 2014. Phytotherapeutic information on plants used for the treatment of tuberculosis in Eastern Cape province, South Africa. Evidence-Based Complementary and alternative Medicine 2014, 1-11.

Madikizela, B., Ndhlala, A.R., Finnie, J.F., Van Staden, J., 2014. Antimycobacterial, anti-inflammatory and genotoxicity evaluation of plants used for the treatment of tuberculosis and related symptoms in South Africa. Journal of Ethnopharmacology 153, 386-391.

Makunga, N.P., Philander, L.E., Smith, M., 2008. Current perspectives on an emergind formal natural products sector in South Africa. Journal of Ethnopharmacology 119, 365-375.

Marrita, R.M., Okemo, P.O., Orodho, J.A., Kirimuhuzya, C., Otieno, J.N., Joseph, M,J., 2010. Efficacy of 13 medicinal plants used by indigenous communities around lake Victoria Kenya, against tuberculosis, diarrhoea causing bacteria and *Candida albicans*. International Journal of Pharmacy & Technology 2(3), 771-791.

Mathabe, M. C., Hussein, A. A., Nikolova, R. V., Basson, A. E., Meyer, J.J.M., Lall, N., 2008. Antibacterial activities and cytotoxicity of terpenoids isolated from *Spirostachys africana*. Journal of ethnopharmacology 116(1), 194-197.

Mativandlela, S.P.N., Meyer, J.J.M., Hussein, A.A., Houghton, P.J., Hamilton, C.J., Lall, N., 2008. Activity against *Mycobacterium smegmatis* and *M.tuberculosis* by extract of South African medicinal plants. Phytotherapy research 22, 841-845.

McBride, J., Ingram, P.R., Henriquesz, F.L., Roberts, C.W., 2005. Development of colometric microtiter plate assay for assessment of antimicrobials against Acanthamoeba. Journal of Clinical Microbiology, 629-634.

McGaw, L.J., Lall, N., Meyer, J.J.M., Eloff, J.N., 2008. The potential of south African plants against Mycobacterium infections. Journal of Ethnopharmacology 119, 482-500.

Morens, D.M., Fauci, A.S., 2012. Emerging infectious disease in 2012: 20 Years after the institute of medicine report. Perspective 3(6), 454-461.

Mosmann, T.,1983. Rapid colometric assay for cellular growth and survival: application to proliferation and Cytotoxicity assays. Journal of Immunological methods 65 (1-2), 55-63.

Naidoo, D., Maharaj, V., Crouch, N.R., Ngwane, A., 2011. New labdane-type diterpenoids from *Leonotis leonurus* support circumscription of Lamiaceae. Biochemical Systematics and Ecology 39 (2009), 216-219.

Newton, S.M., Lau, C., Gurcha,S.S., Besra, G,S., Wright,C.W., 2002. The evaluation of forthy-three plant species for *in vitro* antimycobacterial activities; isolation of active constituents from Psoralea corylifolia and Sanguinaria Canadensis. Journal of Ethnopharmacology 79, 57-67.

© University of Pretoria

Nielson, T.R.H., Kuete,V., Jager,A.K., Meyer, J.J.M., Lall, N., 2012. Antimicrobial activity of selected South African medicinal plants. BMC Complementary and Alternative Medicine 12 (74), 1-6.

O'Brien, J., Wilson, I., Orton, T., Pognan, F., 2000. Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry 267, 5421–5426.

Ogbole, O.O., Ajaiyeoba, E.O., 2009. Traditional management of tuberculosis in Ogun state of Nigeria: The practice and Ethnobotanical survey. African Journal of Traditional Complementary and alternative Medicines 7 (1), 79-84.

Protocol online.org. 2005. Problems with U937. Available at: http://www.protocol-online.org/biology-forums/posts/6696.html (Accessed 07 December 2012).

Roche.2005. Cell Proliferation Kit II (XTT). CAT number: 11 465 015 001.

Sarker, S.D., Nahar, L., Kumarasamy, Y., 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods 42, 321-324.

Sharp,B.M., 2013. Conversion of the U937 momocyte into "Macrophage-like" populations exhibiting MI or M2 characteristics. Wright state University, Masters thesis.

Siddique, A. A., Joshi, P., Misra, L., Sangwan, N. S., Darokar, M. P., 2014. 5, 6-De-epoxy-5-en-7-one-17-hydroxy withaferin A, a new cytotoxic steroid from *Withania somnifera* L. Dunal leaves. Natural product research 28(6), 392-398.

Sigma-aldrich. 2012. Online. Product information, Actinomycin D. available at: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Datasheet/6/a1410dat.Par.0001.File.tmp/a1410dat.pdf (Accessed 07 December 2013).

Silva, A. C. P., de Faria, D. E. P., Santo Borges, N. B. D. E., de Souza, I. A., Peters, V. M., de Oliveira Guerra, M., 2007. Toxicological screening of Euphorbia tirucalli L.: developmental toxicity studies in rats. Journal of ethnopharmacology 110(1), 154-159.

Singh,G., Kumar, P., 2011. Evaluation of antimicrobial efficacy of Flavonoids of *Withania Somnifera* L.. Indian Journal of Pharmacological science 73 (4), 473-478.

Van de Loosdrecht,A.A., Beelen, R.H.J., Ossenkoppele, G.J., Broekhoven, M.G., Langehuijsen, M.M.A.C., 1994. A tetrazolium-based colometric MTT assay to quantitate human monocytes meadited Cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. Journal of Immunological Methods 174, 311-320.

WebMD. 2005. Online. What is Tuberculosis? Available at: http://www.webmd.com/a-to-z-guides/understanding-tuberculosis-basics (Accessed 15 September 2014).

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Chapter 4

Inhibitory effects of the selected medicinal plant extracts against Glutathione reductase and Mycothiol reductase

The plant extracts were further tested for their activity against Glutathione disulfide reductase (Human analogue) and Mycothiol disulfide reductase (*Mycobacterium* analogue). The inhibitory activity of the plant extracts was determined using a DTNB-coupled Glutathione/Mycothiol disulfide reductase assay. *Typha minima* showed a potential activity against Mtr with an EC_{50} of 47.89±47.5 and had a less activity against Gtr.

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4. Background

The plant extracts were evaluated for their mechanism of action against Glutathione disulfide reductase (Human analogue) and Mycothiol disulfide reductase (Mycobacterium analogue).

4.1 Lineage of Glutathione & Mycothiol reductase

The flavoprotein disulfide reductase (FDR) family of enzymes have high sequence and structural homology. The first enzymes to be included in this family were lipoamide dehydrogenase (LipDH), thioredoxin reductase (TrxR) and glutathione reductase (GR). In the 1980`s another enzyme known as trypanothione reductase (TR) was discovered in trypanosome spp. and added into this family. Recently Mycothiol reductase (Mtr) from *Mycobacterium tuberculosis* was also added into the mentioned enzyme family. These homodimeric flavoproteins consists of flavin adenine dinucleotide (FAD) and one redox-active disulfide which are bound together and used to catalyze the pyridine-nucleotide-dependent reduction of their disulfide-bonded substrates. The similarity of these enzymes was confirmed by cloning and DNA sequencing of the genes encoding the enzymes and three dimensional structure determinations using X-ray crystallography. The enzymes of interest for this project are glutathione reductase (GR) which is the human analogue and Mycothiol reductase (Mtr) which is the *Mycobacterium* analogue (Pai & Schulz, 1983, Arscott, 1997, Zhong *et al*., 1997, Argyrou and Blanchard, 2004).

4.1.1 The molecular structures of Glutathione and Mycothione reductase

Glutathione reductase is a ubiquitous enzyme required for the conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH). This enzyme is categorised under the name of disulfide oxidoreductases which catalyse the pyridine-nucleotide-dependent reduction of different types of substrates including disulfide-bonded substrates. All the glutathione reductase family members share a similar three-dimensional structure in their FAD binding domain and a sustained sequence motif (Figure 4.1). The topology of the GR family consists of a central five-stranded β-sheet (β_1, β_2, β_3, β_7 and β_8) surrounded by α-helices (α_1 and α_2) and an additional crossover connection composed of a three-stranded antiparallel β-sheet ($\beta_{4\text{-}6}$). Glutathione reductase is a homodimeric enzyme of which each subunit contains four well-defined domains. The dimeric nature is crucial for its function, because both subunits contribute with essential residues to the constitution of the active site (Mittl and Schulz, 1994).

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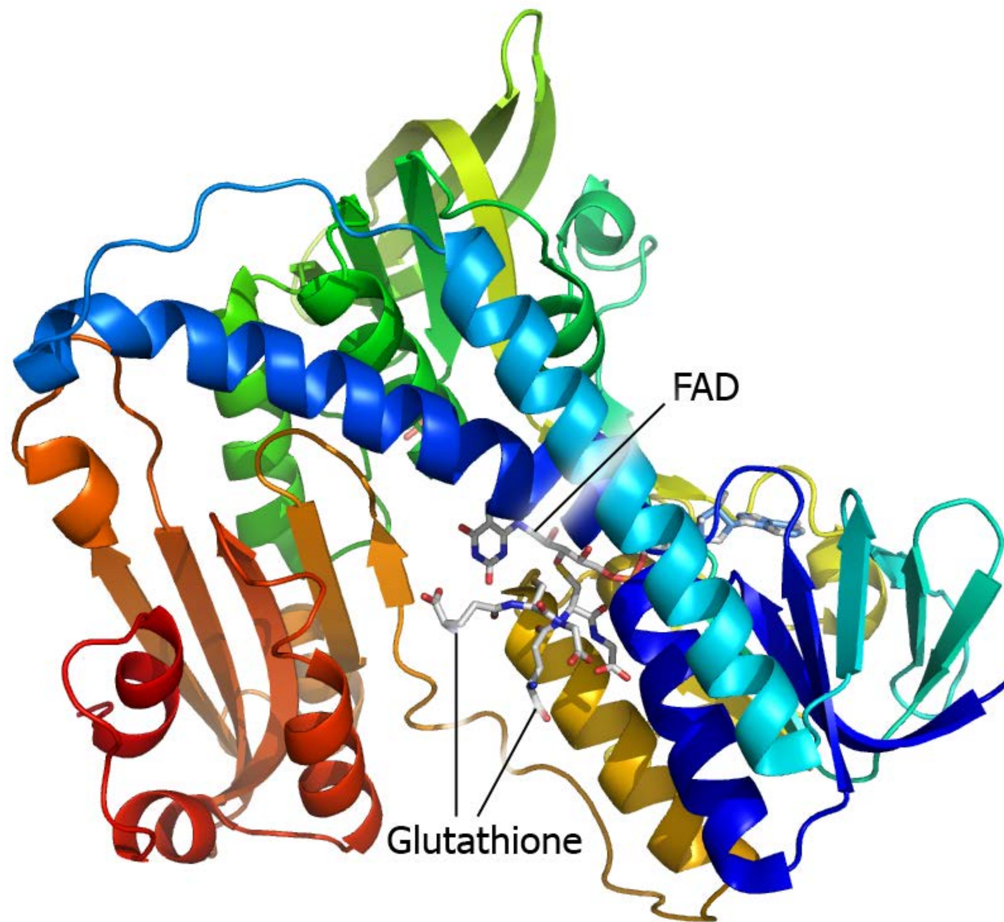

Figure 4.1: The structure of Glutathione reductase (Mittl and Schulz, 1994)

4.1.2 The function of Glutathione and Mycothione reductase

Reactive oxygen species (ROS), are the by products generated from essential oxygen cellular metabolism (Yu, 1994). The by products act as a defence mechanism against pathogens (Yan *et al*., 2013) in the cell but a high amount of these can be detrimental to cellular homeostasis. There are molecules employed for elimination of ROS in cells. Glutathione (GSH) found in eukaryotes and gram-negative bacteria (Rawat and Av-Gay, 2007), is responsible for the eradication of ROS such as H_2O_2, through an enzymatic reaction catalysed by glutathione reductase (GR) which reduces glutathione from its oxidised form (GSSG) to (GSH) in the presence of NADPH (Figure 4.2) (Zhu *et al*., 2013).

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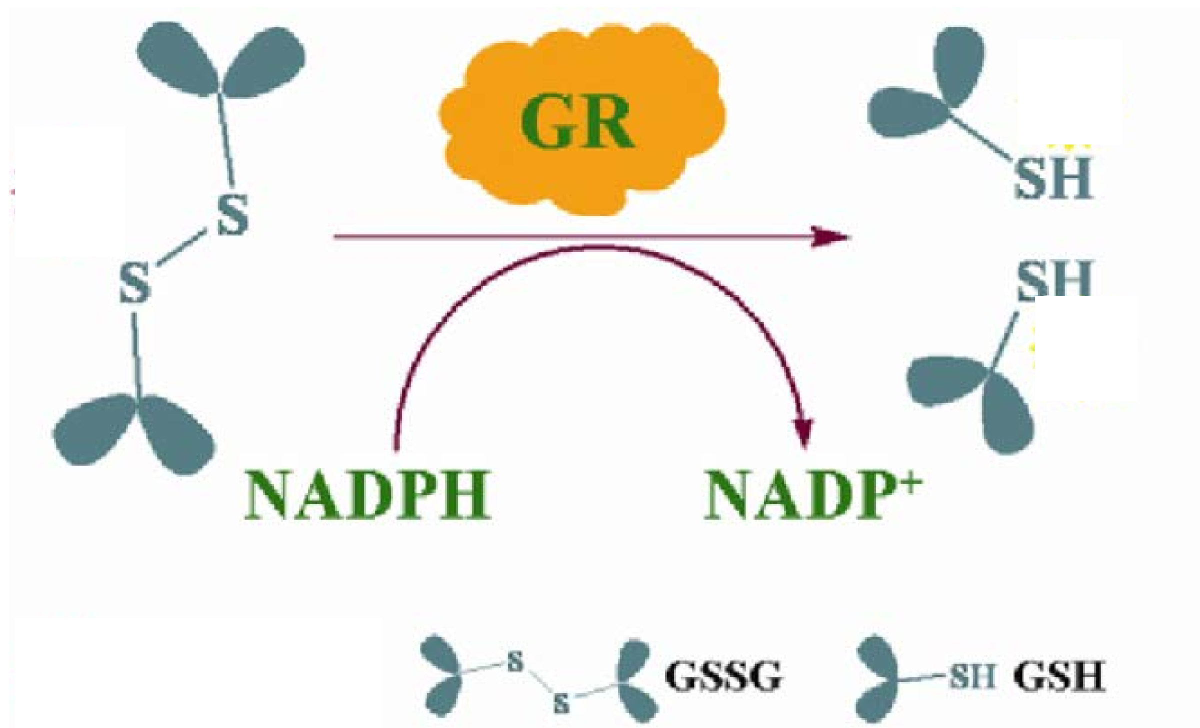

Figure 4.2: An illustration of the reduction of GSSG to GSH (Zhu *et al*., 2013)

Mycobacterium cells lack glutathione which means there is a mycothione specific reductase found in the cells of this bacterium. Mycothione reductase is an analogous form of glutathione reductase predominantly found in actinomycete (Patel & Blanchard, 1999). Mycothione reductase has a function similar to glutathione reductase, it catalyses the reduction of mycothiol in the presence of NADPH, which contributes to the maintaining of intracellular homeostasis in the cells (Patel & Blanchard, 2001, Rawat & Av-Gay, 2007).

4.1.3 Glutathiol and Mycothiol

The tripeptide glutathiol (GSH) is the major low molecular weight thiol found in gram-negative bacteria and most eukaryotes. It serves as a buffer against free radicals and as a reductant of aberrant disulfide bonds that form between small thiol-containing molecules. GSH plays an essential role in protecting the cell against toxic oxygen species through the elimination of reactive oxygen species which are produced in the atmosphere by basil metabolic activities in aerobic organisms. Mostly in higher organisms and gram-negative bacteria GSH can detoxify exogenous xenobiotic agents and participate in the synthesis of metabolites e.g. steroids and prostaglandins. It plays a part in the growth of gram-negative bacteria by acting as a cofactor in various degradation pathways. GSH is also important in the synthesis of deoxyribonucleotides. Glutathione is sustained at high levels in the cells in a form of dimeric glutathione disulfide (GSSH) which is reduced to GSH by an NADPH-

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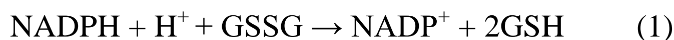
dependent enzyme known as glutathione disulfide reductase (Gtr) (Anderson, 1998; Sheehan *et al*., 2001; Vuilleumier and Pagni, 2002; Pastore *et al*., 2003 Deponte, 2013; Morris *et al*., 2013). The reaction can be seen in equation 1 (Eq. 1)



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

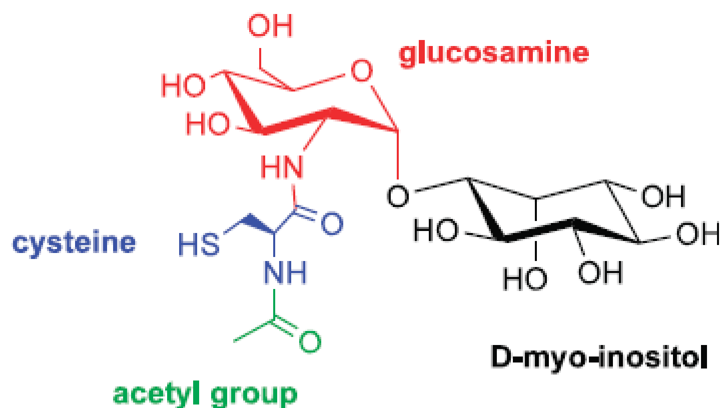


Figure 4.3: Structure of Mycothiol (Rawat and Av-Gay, 2007)

Most gram-positive bacteria such as actinomycetes ; *Mycobacterium* lack GSH and instead have an analogue known as 1-D-myo-inositol-2-(*N*-acetyl-L-cysteinyl) amido-2-deoxy-α-D-glucopyranoside commonly known as mycothiol (MSH), which is a low- molecular weight thiol or protein which is present in millimolar amounts (high levels of accumulation in *Mycobacterium* and *Streptomyces*). Mycothiol has been isolated from *Streptomyces clavuligerus* and *Mycobacterium bovis* (Newton *et al.,* 1995). MSH is found in seven subgroups of the actinomycetes (Newton and Fahey, 2002). MSH is an antioxidant in actinomycetes analogous to that of GSH (Newton and Fahey, 2002). The thiol plays a role in the detoxification formaldehyde by acting as a cofactor for NAD-dependent formaldehyde dehydrogenase which is thought to detoxify formaldehyde in *Amycolatopsis methanolica* and has the ability to detoxify alkylating agents by converting them to an S-conjugate of MSH. *Mycobacterium tuberculosis* relies on the presence of MSH in order to survive and to possess pathogenicity leading to the organism being able to withstand harsh environmental conditions as well as antibiotics (Buchmeier *et al*., 2003; Anderberg *et al*., 1998; Knapp *et al*., 2005; Fan *et al*., 2009; Holsclaw *et al*., 2011).

An amide bond linking acetyl group to cysteine and a second amide bond between cysteine and glucosamine are characteristic of MSH (Figure 4.3). GSH has two amino acids namely, glycine and glutamic acid which are not present in MSH instead it has two sugar moieties,

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inositol and *N*-glucosamine (Figure 4.3). The evolution of MSH as a major intracellular thiol in actinomycetes could be due to the sustainability of the bonds (Newton *et al.,* 1995). MSH is resistant to autoxidation due to the presence of cysteine which undergoes heavy metal catalysed autoxidation much more rapidly than GHS, resulting in the formation of toxic peroxy radicals and hydrogen peroxide which is lethal to cells (Rawat and Av-Gay, 2007). Cysteine needs to be maintained at low concentration as it is a liability. Another form of auto-oxidation that MSH undergoes is copper catalysed auto-oxidation which is 30-fold slower than that of cysteine and 7-fold slower than that of GSH. These differences contribute to the ability of actinomycetes to cope better with oxidative stress as compared to gram-negative bacteria and eukaryotes. The absence of a metal chelating agent could also contribute to MSH being more resistant to auto-oxidation (Newton *et al*., 1995; Newton *et al*., 2008; Deponte, 2013).

4.1.3.1 Mycothiol biosynthesis

Five enzymes are involved in the synthesis of MSH intermediates during biosynthesis of MSH in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis.* MshA, the MSH glycosyltransferase; MshA2, the MSH Phosphatase; MshB, the MSH Deacetylase; MshC, the MSH Ligase and MshD, the MSH Synthase (McCarthy *et al*., 2004;Rawat and Av-Gay, 2007;Newton *et al*., 2008; Holsclaw *et al*., 2011).

Genes encoding MSH biosynthetic enzymes have been determined through the mutation of genes required for MSH production. These principles were used to determine the genes that play a pivotal role in MSH biosynthesis and in identifying the different functions within the cell that require MSH (Newton *et al*., 2008). The studies were initially conducted on *M. smegmatis* which is a genetic homologue to *M. tuberculosis*. Mutants of the MSH biosynthetic enzymes except for MshA2 were produced. Upon analysis it was determined that MSH mutants have an increased sensitivity to reactive oxygen and nitrogen species, alkylating agents and antibiotics. Therefore, MSH is required in multiple detoxification mechanisms (Rawat *et al*., 2002; Newton *et al*., 2008). This indicates the crucial role that MSH plays in the growth and pathogenicity of the bacterium (Fan *et al*., 2009).

An interesting observation was the >25-fold increased resistance of the mutants to isoniazid and ethionamide which are anti-TB drugs. The high levels of resistance of the MSH mutants to INH and ethionamide suggest that MSH is involved in the activation of these drugs. The MSH mutants showed an increased resistance to antibiotics such as rifamycin, streptomycin,

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erythromycin and azithromycin (Newton *et al*., 2008). The same principles were applied to *M. tuberculosis* in order to determine the role of MSH in maintaining its viability and the results indicate that the survival of *M. tuberculosis* in environments that constitute reactive oxygen and reactive nitrogen intermediates within macrophages requires MSH-dependent systems (Newton *et al*., 2008).

Mycothione reductase was one of the first enzymes to be characterised as responsible for MSH metabolism. The main function of glutathione and mycothione reductase enzymes is to act as buffers maintaining GSH and MSH in a highly reduced state, in order to ensure the defence against free radicals and antioxidants is sustained (Yan *et al*., 2013).

4.1.3.2 Mycothiol as a potential drug target

Mycothiol has been viewed as potential drug target due to its novel structure and being the predominant thiol in *Mycobacterium* (Patel & Blanchard, 2001; McCarthy *et al*., 2004). Many enzymes are involved in the biosynthesis of MSH and all these enzymes have been considered as drug targets (Holsclaw *et al*., 2011). The targets need to meet a certain criteria in order to qualify as drug targets; inhibitors must be able to bind to the active site. Many drugs have been implemented to eradicate *M. tuberculosis* but have certain limitations they are not able to kill the bacterium in the latent or dormant stage of growth (Newton *et al*., 2008).

This has led to the investigation of new anti-TB drugs which are effective against latent *M.tuberculosis*. It has been shown that the inhibition of MSH synthesis leads to inactivation of metabolic processes that require the presence of MSH in the bacterium. Enzymes involved in the metabolism of MSH such as Mtr have been considered as possible drug targets. The importance of Mtr in the growth of *M.tuberculosis* has not been well established. Previous studies show that Benzo- and naphthoquinone derivates are reduced by Mtr and can serve as subversive substrates by enhancing redox cycling (Newton *et al*., 2008). What makes Mtr an important potential drug target is the fact that the inhibition of Mtr results in decreased levels of reduced MSH in the cell therefore increasing the sensitivity of the bacterium to toxins and antibiotics. MSH has an effecton the susceptibility of the bacterium to anti-tuberculosis drugs such as isoniazid and rifampin (Nilewar & Kathiravan, 2014).

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4.1.4 Rational statement

Due to the limitations associated with first and second line drugs against tuberculosis, alternative sources of anti-TB drugs, such as plants have been investigated. Plant extracts were analysed to determine if they have inhibitory activity against Mtr using enzyme assay.

4.2 Materials & Methods

4.2.1 Plant extracts

Twenty medicinal plant extracts were screened for any inhibitory properties against Gtr and Mtr enzymes

4.2.2 DTNB-coupled Glutathione disulfide reductase assay

The assay is based on the reaction which occurs in organisms such as humans under normal conditions which results in the production of GSH which is responsible for the protection of the organism against reactive oxygen species and plays a role in diseases, such as arthritis, inflammation and aging (Baker *et al*., 1990). The assay was firstly introduced by Owen and Belcher in 1965 and later modified by Tietze by incorporating DTNB into the reaction in order to increase the sensitivity of the spectrophotometers procedure and by analyzing the enzymatic reaction in a microtitre plate reader. Owen and Belcher defined it as a rapid colometric and apparently specific micro-method. In this study the assay was modified to determine if the plant samples can bind to the Gtr and inhibit the enzymatic reaction which results in the production of GSH.

4.2.2.1 Experimental procedure

Dimethyl-sulfoxide (DMSO), NADPH and glutathione disulfide reductase (Gtr) were purchased from Sigma. Inhibitory assays of the 20 medicinal plant extracts on Gtr were carried out at 30^0C in 110µl of 50mM HEPES containing 0.1 mM EDTA at a pH of 7.6. NADPH was at a concentration of 140µM, DTNB at a concentration of 100µM and GSSG (substrate) at a concentration of 60µM. The assay for each plant sample was conducted in a 96-well microtitre plate. The assay was carried out at seven different concentrations (two-fold dilution). In each well was added 20µl of Gtr, 20µl NADPH, 20µl DTNB, 110µl Buffer, 10µl plant sample which was dissolved in 100% DMSO. The reaction was initiated by the addition of the substrate GSSH. Each well contained a final assay volume of 200µl. The plates were read using a micro plate reader and the data was processed using two computer programmes: KC junior™ for data capturing and Graph pad prism™ programme for analysis.

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4.2.3 DTNB-coupled Mycothione disulfide reductase

This assay is based on the same principles as glutathione reductase inhibitory assay with some differences (Figure 4.4). Mycothiol disulfide (MSSH) is used as the substrate, mycothiol disulfide reductase (Mtr) is used as the enzyme for the reaction. Due to the limited availability of MSSH substrate, the product MSH was mixed with DTNB in order to react with it and oxidize MSH to MSSH prior to adding Mtr for the start of the enzymatic reaction (Hamilton *et al*., 2009).

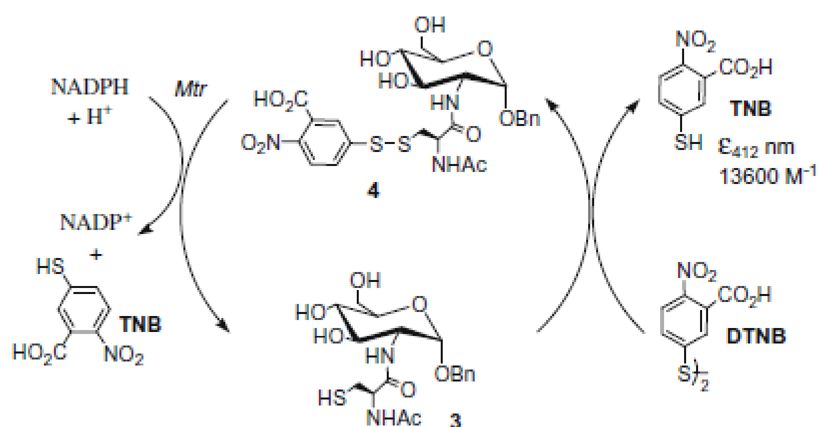

Figure 4.4: A schematic presentation of DTNB-mycothiol disulfide reductase assay, 3; MSH, 4; MSSH (Hamilton *et al*., 2009).

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4.3 Results & Discussion

Table 4.1: Twenty medicinal plant extracts were screened for any inhibitory properties against Gtr and Mtr enzymes

Plant extract	Gtr[a] EC_{50} [c] (µg/ml)	Mtr[b] EC_{50} [c] (µg/ml)
Alectra sessiliflora	710.2±4240	167.8±449.04
Aloe plicatilis	954961±273.3	993.4±1833.05
Cassinopsis ilicifolia	NA[d]	3.697±9456
Dracaena aletriformis	10214±924.1	0.0004655±1.88
Dracaena draco	14976±104.1	4688±0.28
Eucomis autumnalis ssp. clavata	NA	NA
Eucomis humilis	1303±2219.8	320.6±359.1
Eucomis vandermerwei	**22.31±79.64**	134.7±3489
Euphorbia tirrucalli	258.7±400	154±229.6
Faurea saligna	4608±151153.385	147.7±58.85
Ficus sur	**44.47±8.425**	NA
Ficus sycomorus	967.3±1247	17426±0.55
Leonotis leonurus	**3.461±812.2**	1261±2.353
Merwillia plumbea	256.3±3.167	236.1±195.75
Salvia africana-lutea	224.1±96.6	NA
Sphedamnocarpus pruriens	**54.27±17.13**	**61.54±16.63**
Tarchonanthus camphorates	65.92±89.58	152.4±2564
Typha capensis	NA	45.19±26.155
Typha minima	**813.5±3.21**	**47.89±47.5**
Withania somnifera	221.2±122.9	162.4±351.6

[a] Glutathione disulfide reductase

[b] Mycothiol disulfide reductase

[c] Fifty percent effective concentration.

[d] Not available (inconclusive readings)

The plant extracts were analysed if they had inhibitory activity against Glutathione disulfide reductase (Human analogue) and Mycothiol disulfide reductase (Mycobacterium analogue) enzymes. Effective concentration at which 50% activity is inhibited (EC_{50}) was used to

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establish activity. A plant extract with an EC_{50} lower than 50µg/ml was considered to have activity against a particular enzyme. Ideally a plant extract with high affinity for Mtr and low affinity for Gtr would be considered a good candidate. *Typha minima* had a higher affinity for Mtr (47.89µg/ml) and less affinity for Gtr (813.5µg/ml) (Table 4.1). The phytochemical makeup; consisting of phenols, alkaloids, tannins, Flavonoids can be the reason for inhibitory activity against Mtr (Londokar *et al*., 2013).

Sphedomnocarpus pruriens had a similar affinity for both enzymes, which could indicate similar effect in both humans and bacteria. *Eucomis vandermerwei*, *Ficus sur* and *Leonotis leonurus L.* had a high affinity for Gtr and low affinity for Mtr. In this case it indicates their toxicity to humans as by inhibiting Gtr, which can result in increased levels of reactive oxygen species in human cells as a result of decline of glutathione. Based on these results the aforesaid plants extracts might not be considered as candidates for inhibitory activity against Mtr.

A pure isolated flavones compound, 5, 7, 2`-trihydroxyflavone from *Galenia africana* was tested to determine if it can act on Mtr as a subversive substrate. The results showed that the compounds failed to bind as a substrate on Mtr which means Mtr is not a target of the flavones. The flavones could possibly target other flavoproteins oxidoreductases (Mativandlela *et al*., 2008). It has been reported that Naphthoquinones which are widely distributed in plants have pharmacological properties including antibacterial, antiviral, anticancer, antimalarial and antifungal activity and can act on Mtr as a subversive substrate. A naphthoquinone 7-methyljuglone (5-hydroxy-7-methyl-1, 4-naphthoquinone) was isolated from *Euclea natalensis* and has shown antimycobacterial activity against *M.tuberculosis*. Naphthoquinones can act as subversive substrates (substitute the necessary substrate and allow the enzyme to reduce it) (Mahapatra *et al*., 2007). It is regenerated by inhibiting the normal reduction of the original substrate such as MSSH in mycobacterium therefore interfering with normal occurrence of the enzymatic reaction. In the present study crude plant extracts were tested for inhibitory activity whereas in the reported studies pure compounds were used, which can contribute to the distinction in the results obtained.

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4.4 Conclusion

The study of the mechanism of action of the plant extracts was conducted with a focus on the glutathione disulfide reductase and mycothiol disulfide reductase enzymes. One plant extract, *Typha minima* showed potential activity by binding to Mtr and having less affinity for Gtr. The phytochemical constituents could play a role in this as antimicrobial activity of this plant has been reported. This sample can be further analysed as to whether the activity is a result of a mono-compound or multiple compounds acting synergistically. Pure compounds seem to be effective in inhibiting the enzymes as compared to crude plant extracts.

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4.5 References

Anderberg, S. J., Newton, G. L., Fahey, R. C., 1998. Mycothiol Biosynthesis and Metabolism Cellular levels of potential intermediates in biosynthesis and degradation of Mycothiol in *Mycobacterium smegmatis*. Journal of Biological Chemistry 273(46), 30391-30397.

Anderson, M.E., 1998. Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111-112, 1-14.

Argyrou, A., Blanchard, J.S., 2004. Flavoprotein disulfide reductases: advances in chemistry and function. Progress in Nucleic Acid Research and Molecular Biology, 89-142.

Arscott, L., 1997. The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from *Escherichia coli*. Proceedings of the National Academy of Sciences 94.8 (1997), 3621-3626.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 190, 360-365.

Buchmeier,N.A., Newton, G.L., Koledin, T., Fahey,R.C., 2003. Association of mycothiol with protection of Mycobacterium tuberculosis from toxic oxidants and antibiotics. Molecular Microbiology 47, 1723-1732.

Deponte, M., 2013. Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. Biochimica et Biophysica Acta (BBA)-General Subjects, 1830(5), 3217-3266.

Fan, F., Vetting, M.W., Frantom, P.A., Blanchard, J.S., 2009. Structure and mechanisms of the mycothiol biosynthetic enzymes. Current Opinion in Chemical Biology 13, 451-459.

Hamilton, C.J., Finlay, R.M.J., Stewart,M.J.G., Bonner, A., 2009. Mycothiol disulfide reductase: A continuous assay for slow time-dependent inhibitors. Analytical Biochemistry 388, 91-96.

Holsclaw, C. M., Muse, W. B., Carroll, K. S., Leary, J. A., 2011. Mass spectrometric analysis of mycothiol levels in wild-type and mycothiol disulfide reductase mutant *Mycobacterium smegmatis*. International Journal of mass spectrometry 305(2), 151-156.

Knapp, S., Amorelli, B., Darout, E., Ventocilla, C. C., Goldman, L. M., Huhn, R. A., Minnihan, C.,2005. A family of mycothiol analogues. Journal of carbohydrate chemistry 24(2), 103-130.

Londokar, R. L., Kattegouga, U. M., Shivsharanappa, K., Hanchinalmath, J. V., 2013. Phytochemical screening and *in vitro* antimicrobial activity of *Typha angustifolia*

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Linn leaves extract against pathogenic gram negative micro organisms. Journal of pharmacy research 6(2), 280-283.

Mahapatra, A., Mativandlela,S.N.P., Binneman, B., Fourie, P.B., Hamilton, C.J., Meyer, J.J.M., van der Kooy, F., Houghton, P., Lall, N., 2007. Activity of 7-methyljuglone derivatives against *Mycobacterium tuberculosis* and as subversive substrates for mycothiol disulfide reductase. Bioorganic & Medical Chemistry 15, 7638-7646.

Mativandlela, S.P.N., Meyer, J.J.M., Hussein, A.A., Houghton, P.J., Hamilton, C.J., Lall, N., 2008. Activity against *Mycobacterium smegmatis* and *M.tuberculosis* by extract of South African medicinal plants. Phytotherapy research 22, 841-845.

McCarthy, A. A., Peterson, N. A., Knijff, R.,Baker, E. N., 2004. Crystal structure of MshB from *Mycobacterium tuberculosis*, a deacetylase involved in mycothiol biosynthesis. Journal of molecular biology 335 (4), 1131-1141.

Mittl, P.R.E., Schulz, G.E., 1994. The structure of glutathione reductase from *Escherichia coli* at 1.86 angstroms resolution: comparison with the enzyme from human erythrocytes. Protein Scientific 3, 799-809.

Morris, D., Khurasany, M., Nguyen, T., Kim, J., Guilford, F., Mehta, R., Venketaraman, V., 2013. Glutathione and infection. Biochimica et Biophysica Acta (BBA)-General Subjects, 1830(5), 3329-3349.

Newton, G.L., Bewley,C.A., Dwyer,T.J., Horn, R., Aharonowitz, Y., Cohen, G., Davies, J., Faulkner, D.J., Fahey, R.C., 1995. The structure of U17 isolated from *Streptomyces clavuligerus* and its properties as an antioxidant thiol. European Journal of Biochemistry 230, 821-825.

Newton, G.L., Buchmeier, N., Fahey, R.C., 2008. Biosynthesis and functions of mycothiol, the unique protective thiol of Actinobacteria. Microbiology and molecular biology reviews 72 (3), 471-494.

Newton, G.L., Fahey, R.C., 2002. Mycothiol biochemistry. Archives of Microbiology 178 (6), 388-394.

Nilewar, S.S., Kathiravan, M.K., 2014. Mycothiol: A promising antitubercular target. Bioorganic chemistry 52, 62-68.

Pai, E. F., and Georg E. Schulz., 1983. The catalytic mechanism of glutathione reductase as derived from x-ray diffraction analyses of reaction intermediates. Journal of Biological Chemistry 258.3, 1752-1757.

Pastore, A., Federici, G., Bertini, E., Piemonte, F., 2003. Analysis of glutathione: implication in redox and detoxification. Clinica chimica acta, 333(1), 19-39.

Patel, M. P., Blanchard, J. S., 1999. Expression, purification, and characterization of *Mycobacterium tuberculosis* mycothione reductase.Biochemistry, 38(36), 11827-11833.

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Patel, M. P., Blanchard, J. S., 2001. *Mycobacterium tuberculosis* mycothione reductase: pH dependence of the kinetic parameters and kinetic isotope effects. Biochemistry 40(17), 5119-5126.

Rawat, M., Av-Gay, Y., 2007. Mycothiol-dependent proteins in actinomycetes. Federation of European Microbiological Societies Microbiological reviews 31, 278-292.

Rawat, M., Newton, G. L., Ko, M., Martinez, G. J., Fahey, R. C., Av-Gay, Y., 2002. Mycothiol-deficient *Mycobacterium smegmatis* mutants are hypersensitive to alkylating agents, free radicals, and antibiotics. Antimicrobial agents and chemotherapy 46(11), 3348-3355.

Sheehan, D., Meade, G., Foley, V.M., Dowd, C.A., 2001. Structure, function and evolution of glutathione transferases: implications for classification of non- mammalian members of an ancient enzyme superfamily. Biochemistry Journal 360, 1-16.

Vuilleumier, S., Pagni, M., 2002. The elusive roles of bacterial glutathione S-transferases: new lessons from genomes. Appl Microbiol Biotech 58, 138-146.

Yan, J., Ralston, M. M., Meng, X., Bongiovanni, K. D., Jones, A. L., Benndorf, R., 2013. Glutathione reductase is essential for host defense against bacterial infection. Free Radical Biology and Medicine (61), 320-332.

Yu, B. P., 1994.Cellular defenses against damage from reactive oxygen species. Physiological reviews 74.1, 139-162.

Zhong, L., Arne´ r‡, E.S.J., Ljung, J., Aslund, F., Holmgren, A., 1997. Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. Journal of Biological Chemistry 273.15, 8581-8591.

Zhu, S., Zhao, X. E., Zhang, W., Liu, Z., Qi, W., Anjum, S., Xu, G., 2013. Fluorescence detection of glutathione reductase activity based on deoxyribonucleic acid-templated silver nanoclusters. Analytica chimica acta (786), 111-115.

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Chapter 5

Inhibition of biofilm formation by medicinal plant extracts

The 20 plant extracts were screened to check whether they had antimicrobial and antibiofilm formation activity. Utilizing *Mycobacterium smegmatis* as the test organism, which is a close relative to *Mycobacterium tuberculosis*. No significant antimicrobial activity was observed from the plant extracts. Qualitatively the plant extracts; *Sphedamnocarpus pruriens* (N), *Salvia africana lutea* (L), *Withania somnifera* (R) showed considerable antibiofilm activity but was validated by determining quantitavely. The effective dose (EC50) was determined and *Leonotis leonurus* L. (K), *Salvia africana lutea* (L), and *Sphedamnocarpus pruriens* (N) showed potential biofilm formation inhibition of 45.55±0.2475, 100±56.83 and 61.39±60.59 respectively.

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5. Background

Most of the bacteria develop a resistance to primary inhibitors or ailments, therefore bacterial defence has evolved. Most first and second line drugs used against *Mycobacterium tuberculosis* have become effective. Due to an extracellular matrix layer that is impermeable to most drugs; biofilm is used a as defence mechanism for *Mycobacteria*. Apart from analysing whether the South African medicinal plants have antimycobacterial activity in a planktonic form, there is a need to determine if the medicinal plant extracts can penetrate or disrupt the impermeable barrier formation.

5.1 Mycobacterium biofilm

Mycobacteria contain thick rigid cell walls containing large amounts of diverse lipids such as mycolic acid; a defining feature of *mycobacterium* which result in this bacterium having an impermeable, hydrophobic cell envelope (Figure 5.1) which makes it difficult for antibiotics, drugs and cell wall destroying enzymes to infiltrate the cell wall. The cell wall components contribute to virulence, persistence within macrophages and modulation of the host immune response. *Mycobacterium* has plasma membrane and peptidoglycan features that are similar to other gram positive bacteria; peptidoglycan is covalently linked to a surrounding network of arabinogalactans which is covered by layers of mycolates. Mycolic acid layer forms an effective barrier by inhibiting the passage of both hydrophilic and hydrophobic compounds which characterizes mycobacteria as resistant towards antibiotics (Udou, *et al*., 1982; Recht *et al*., 2001; Chen *et al*., 2007; Niederweis *et al*., 2010).

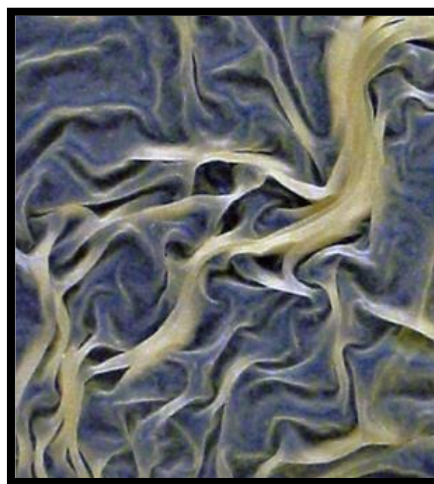

Figure 5.1: Biofilm formation on a liquid surface by *Mycobacterium smegmatis* bacteria (Ostrovsky, 2005)

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5.1.1 Cell wall structure of *Mycobacterium* species

Mycobacteria have an intricate cell envelope structure composed of a cytoplasmic membrane and cell wall (Niederweis *et al*., 2010). These cell envelope characteristics contribute to the permeability barrier. A unique feature of the Mycobacterium cell wall is the cell wall core or rather known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Alderwick *et al*., 2007). This complex is the core of the proper cell wall; which is composed of peptidoglycan, arabinogalactan and mycolic acids (Figure 5.2) (Brennan, 2003; Chatterjee, 1997).

Peptidoglycan is the foundation of the mAGP complex; made up of alternating units of *N*-acetylglucosamine and *N*-glucolylmuramic acid (Besra & Brennan, 1997). *Mycobacterium* peptidoglycan is different from other gram-positive bacteria. Muramic acid residues are glycolylated (Chatterjee, 1997).Arabinogalactan (AG) a middle polymer mainly comprised of D-arabinofuranosyl and D-galactofuranosyl residues links to peptidoglycan through substitution of peptidoglycan muramic acid. (Alderwick *et al*., 2007). Upon analyses of the polymer only a few distinct and specific structural motifs were found as compared to other bacterial polymers which are constituted of repeating units (Chatterjee, 1997). Mycolic acids are comprised of long chained (C70-C90) α-alkyl branched and β-hydroxylated fatty acids. They are attached in tetramers to Arabinan portion of the complex in acylated form (Figure 5.2) .The mAGP complex is vital for *Mycobacterium* viability. (Chatterjee, 1997; Alderwick *et al*., 2007; Barkan *et al*., 2009).

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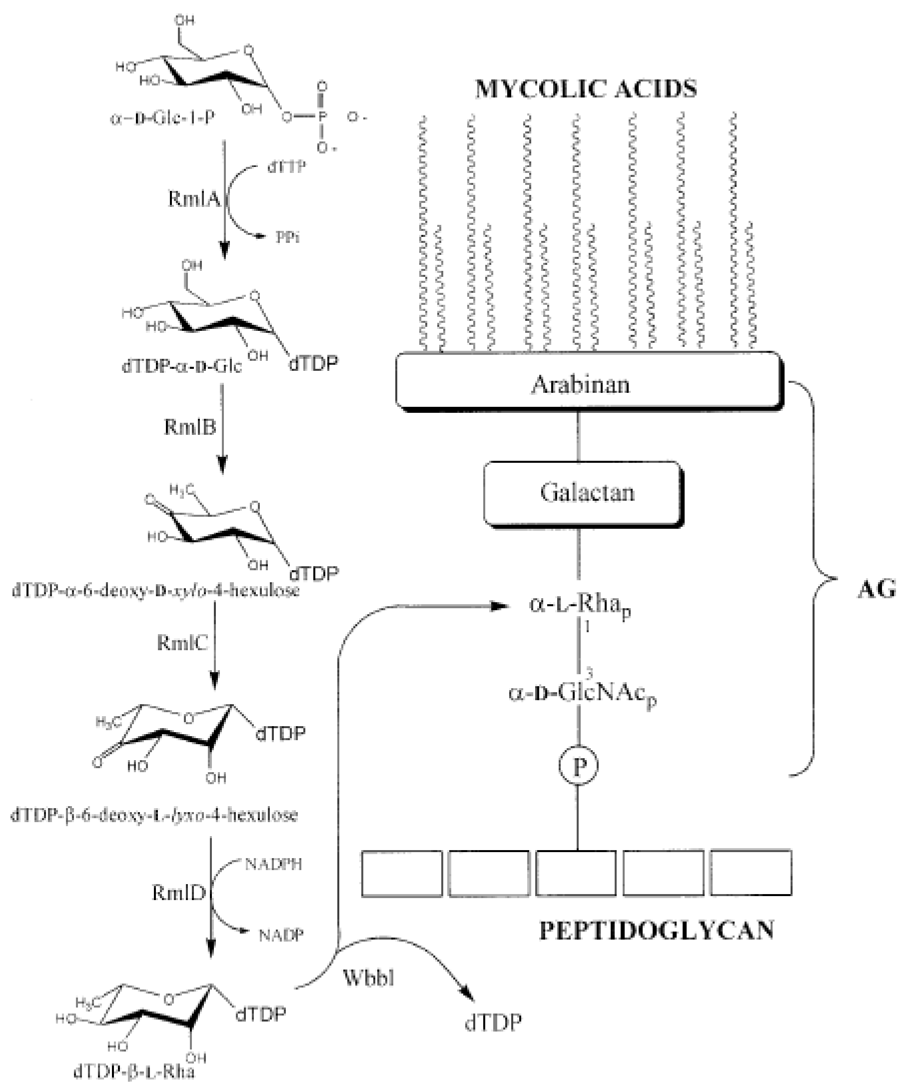

Figure 5.2: Schematic representation of mAGP complex (Ma *et al*., 2001)

5.2 Materials & Methods

5.2.1 Preparation of *Mycobacterium smegmatis* culture

Mycobacterium smegmatis (MC2 155) was kindly sponsored by Medical Microbiology Department from the University of Pretoria; was used as it is a close relative to *Mycobacterium tuberculosis* in terms of their similar virulence gene homologues (Reyrat and Kahn, 2001). The bacterium was cultured onto Middlebrook 7H11 agar base (7H11) and granted growth on the media for 24 hours at 37ºC.

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5.2.1.1 Sample preparation

The ethanol plant extracts were weighed out at a concentration of 2 mg and dissolved in 100% DMSO to get a final concentration of 4000µg/mL of the stock solution. A 5x dilution in 7H9 (10% OADC, 0.5% glycerol and 0.05% Tween 80) medium was prepared by adding 100µl of the stock solution of each ethanol extract to 400µl of 7H9 medium , making the concentration 800µg/mL; in order to test the samples at a concentration of 200µg/mL. The solution is used in the assay. DMSO was used as control by adding 100µl of 100% DMSO to 400µl of 7H9 media. Ciprofloxacin being the positive control drug control was the tested at a concentration of 0.156mg/mL. In order to test the sample at the highest concentration of 0.156mg/mL the sample was further diluted to obtain the desired concentration of 0.624mg/mL.

5.2.1.2 The micro-dilution screening assay

Screening assays of 20 plant extracts were performed using the 96-well Microtitre plate method (Newton *et al*, 2002).

5.2.1.3 Anti-microbial activity assay

The plant extracts were evaluated for anti-microbial activity on 7H9 broth medium supplemented with 0.05% Tween 80 to inhibit physical biofilm formation. *Mycobacterium smegmatis* ($1x10^4$ colony forming unit (CFU)/0.1ml/well) was inoculated in 96-well plate, and then serially diluted samples were added. The bacteria containing plates were incubated at 37ºC for 4 days. The MIC value for anti-microbial activity was determined using Presto blue indicator (Ishida *et al*., 2011; Lall *et al*., 2013).

5.2.1.4 Biofilm formation inhibition assay

Mycobacterium smegmatis ($1x10^5$ CFU/0.1ml/well) was inoculated into a polystyrene 96-well plate, and then serially diluted samples were added. The bacterium was incubated with the samples at 30ºC for 4 days under stationary conditions which induce biofilm formation. The lowest concentration of visible biofilm formation inhibition (Minimum biofilm formation inhibitory concentration (MBFIC)) was determined visually and also quantitavely by obtaining the Optical Density (OD_{600nm}). (Ishida *et al*., 2011; Cady *et al*., 2012).

Optical density (OD_{600nm}) measurements were done after the plates were put in an oven for 24 hours at 37ºC in order to dry out the planktonic cells, leaving only the biofilm in the wells. Crystal violet (0.01%) was added to the wells as a biomass indicator and incubated for a

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period of 15-20 minutes for proper infiltration into the biofilm. That was followed by extraction of the crystal violet using 95% ethanol. Absorption of the extracted absorbed crystal violet was determined using the Elisa plate reader at wavelength of 600nm.

5.3 Results & Discussion

The selectivity of the plant extracts for antibiofilm activity required the investigation of antimicrobial activity (Ishida *et al*., 2011). Antimicrobial activity was done (Table 5.1).

Table 5.1: Antimicrobial activity of the medicinal plant extracts

Plant extract	MIC [a] (µg/ml)
Alectra sessiliflora	1000
Aloe plicatilis	1000
Cassinopsis ilicifolia	>1000
Dracaena aletriformis	>1000
Dracaena draco	>1000
Eucomis autumnalis ssp. clavata	>1000
Eucomis montana/humilis	>1000
Eucomis vandermerwei	>1000
Euphorbia tirrucalli	>1000
Faurea saligna	>1000
Ficus sur	>1000
Leonotis leonurus	1000
Salvia africana-lutea	1000
Scilla natalensis/Merwillia plumbea	1000
Sphedomnocarpus pruriens	1000
Tarchonanthus camphorates	>1000
Typha capensis	>1000
Typha minima	>1000
Withania somnifera	1000
Ciproflaxicin	0.625

[a] Minimum inhibitory concentration

Antimicrobial activity of the plant extracts was observed at a concentration of 1000µg/ml and higher (Table 5.1). As reported by Ishida *et al* 2011 no significant antimicrobial activity was recorded for the compound Desferrioxamine E even though it displayed significant antibiofilm activity.

Biofilm formation inhibition assay was done to determine whether the plant extracts had antibiofilm activity against *Mycobacterium smegmatis*. The results were analysed qualitatively (Minimum biofilm formation inhibitory concentration) and quantitavely (Crystal violet intercalation). Figure 5.3 shows the visual minimum biofilm inhibition concentration

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(MBFIC) of *Sphedamnocarpus pruriens* from the highest tested concentration of 1000µg/ml. The MBFIC of all the tested plant extracts is depicted in figure 5.3. The MBFIC of the plants extracts are shown in figure 5.4.

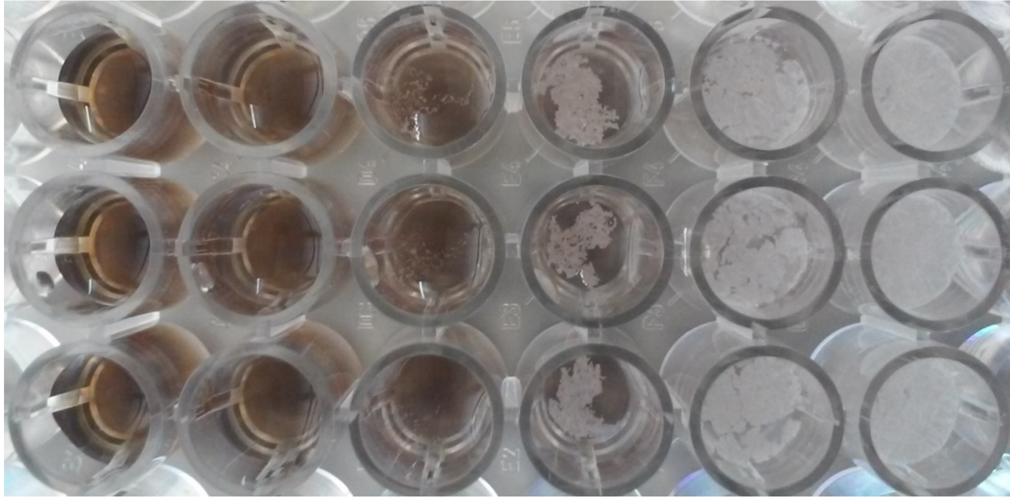

Figure 5.3: Visual representation of *in vitro* gradual Biofilm inhibition by a plant extract

Mycobacterium smegmatis was selected as it has the fastest biofilm formation characteristic compared to other *Mycobacterium* species (Bonkat *et al*., 2012). The extracts of *Salvia africana-lutea* (R), *Sphedamnocarpus pruriens* (N) and *Withania somnifera* (R) showed considerable antibiofilm formation; 421±144.3µg/ml, 585±381.9µg/ml, 334.85±144.3µg/ml respectively when compared to the rest of the extracts (Figure 5.4). There is a significant deviation with the results which depicts that they are not conclusive. The results were further analysed quantitatively using the Elisa plate reader where the optical density (OD_{600}) of the crystal violet/ethanol solution was measured to determine the quantity of biofilm formed (Figure 5.5).

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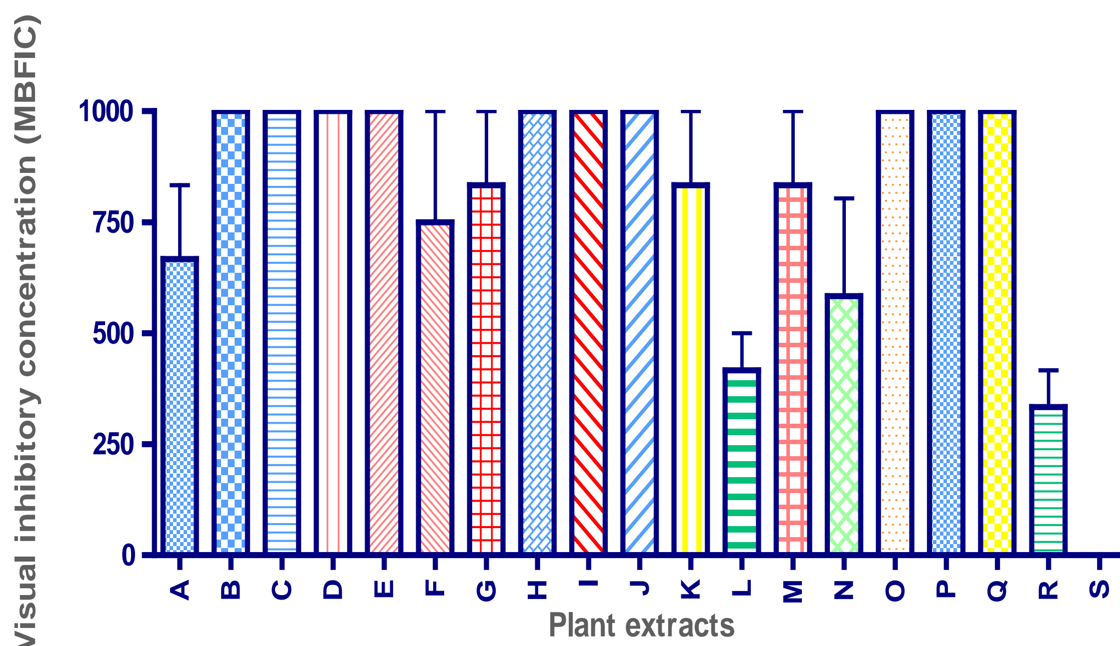

Figure 5.4: Effects of the plant extracts against *Mycobacterium smegmatis* visible biofilm formation

A: *Alectra sessiliflora*
B: *Aloe plicatilis*
C: *Cassinopsis ilicifolia*
D: *Dracaena alectriformis*
E: *Dracaena draco*
F: *Eucomis vandermerwei*
G: *Euphorbia tirucalli*
H: *Faurea saligna*
I: *Ficus sur*
J: *Ficus sycomorus*
K: *Leonotis leonurus*
L: *Salvia africana-lutea*
M: *Merwillia plumbea*
N:*Sphedamnocarpus pruriens*
O: *Tarchonanthus camphorates*
P: *Typha capensis*
Q: *Typha minima*
R: *Withania somnifera*
S: Ciprofloxacin (Positive control)

The graph in figure 5.5 depicts the effective concentration of the plant extracts at which 50% biofilm formation is inhibited. The lower the required concentration the more effective the crude plants extract. Moderate effect was observed from *Alectra sessiliflora* (A), *Eucomis vandermerwei* (F) and *Withania somnifera* (R) with effective doses of 219.80±163.9µg/ml, 263.37±39.63µg/ml and 207.92±151.8µg/ml respectively. Whereas the following plants, *Leonotis leonurus* (K), *Salvia africana-lutea* (L) and *Sphedamnocarpus pruriens* (N) indicated significant effect against biofilm formation with EC_{50} of 45.55±0.2475µg/ml, 100±56.83µg/ml and 61.39±60.59µg/ml respectively. Ciprofloxacin (positive control)

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displayed better inhibitory activity (1.9802µg/ml) when compared to all the plant extracts in this study. This is the first report of afore mentioned plants for antibiofilm activity.

The potential antibiofilm activity of the plant extracts could be due to their chemical constituents. *Salvia Africana-lutea* has been reported to have phenolic compounds such as flavonoids, which have the ability to complex with bacterial cell walls and disrupt microbial membranes by inhibiting matrix formation (Kamatou *et al*., 2005; Cowan, 1999). *Leonotis leonurus and Salvia africana-lutea* are from the Lamiaceae family which means to a certain extent they might have similar chemical compositions therefore supporting the potential of *Leonotis leonurus* for disrupting microbial membranes. *Withania somnifera* has been reported to contain alkaloids; which have antimicrobial activity (Singh *et al*., 2011; Mishra *et al*., 2000). *Sphedamnocarpus pruriens* (Malpighiaceae family) phytochemical compostion has not been reported in literature. Chenthurpandy *et al*., 2009 reported on the phytochemical composition of chloroform and methanol extracts of *Hiptage benghalensis* plant from the Malpighiaceae family. Both the extracts showed presence of flavonoids, which have been reported to bind and inhibit matrix formation (Abidi *et al*., 2014). Since *S.pruriens* is from the same family as *H. benghalensis* the might be similarities in their phytochemical composition. Further phytochemical analysis of these plants must be conducted.

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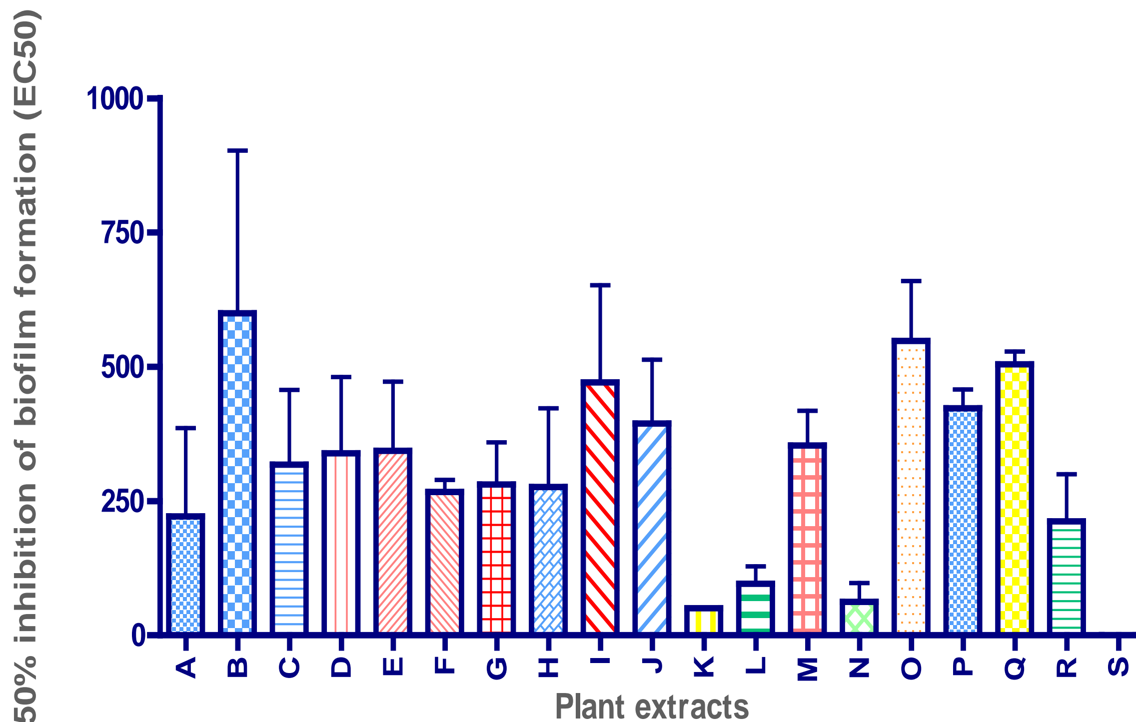

Figure 5.5: Quantitative effects of plant extracts on *Mycobacterium smegmatis* biofilm formation

5.4 Conclusion

The plant extracts were tested for antibiofilm activity. The mechanism of action of the extracts against *Mycobacterium smegmatis* required to determine whether the plant extracts activity is based on what their target on *Mycobacterium* cell wall structure. The chemical composition of *Salvia africana lutea* and *Leonotis leonurus* L. indicated their potential to target biofilm formation based on their ability to disrupt microbial membrane. The plant extracts might have the potentials to disrupt extracellular matrix (mAGP complex) formation. The Lamiaceae family plants and *Sphedamnocarpus pruriens* might have potentials for antibiofilm formation against *Mycobacterium*.

5.5 References

Abidi, S.H., Ahmed, K., Sherwani, S.K., Bibi, N., Kazmi, S.U., 2014. Detection of *Mycobacterium Smegmatis* Biofilm and its control by natural agents. International Journal of Current Microbiology and Applied Sciences 3 (4), 801-812.

Alderwick, L. J., Birch, H. L., Mishra, A. K., Eggeling, L., Besra, G. S., 2007. Structure, function and biosynthesis of the *Mycobacterium tuberculosis* cell wall: arabinogalactan and lipoarabinomannan assembly with a view to discovering new drug targets. Biochemical Society Transactions (35), 1325-8.

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Barkan, D., Liu, Z., Sacchettini, J. C., Glickman, M. S., 2009. Mycolic acid cyclopropanation is essential for viability, drug resistance, and cell wall integrity of *Mycobacterium tuberculosis*. Chemistry & biology 16(5), 499-509.

Besra, G. S., Brennan, P. J., 1997. The mycobacterial cell wall: biosynthesis of arabinogalactan and lipoarabinomannan. Biochemical Society Transactions 25(3), 845-850.

Bonkat, G., Bachmann, A., Solokhina, A., Widmer, A. F., Frei, R., Gasser, T. C., Braissant, O., 2012. Growth of mycobacteria in urine determined by Isothermal microcalorimetry: implications for urogenital tuberculosis and other mycobacterial Infections. Urology 80(5), 1163-1169.

Brennan, P. J.,2003. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis 83(1), 91-97.

Cady, N. C., McKean, K. A., Behnke, J., Kubec, R., Mosier, A. P., Kasper, S. H., Musah, R. A., 2012. Inhibition of biofilm formation, quorum sensing and infection in Pseudomonas aeruginosa by natural products-inspired organosulfur compounds. PloS one, 7(6), 38492.

Chatterjee, D., 1997. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Current opinion in chemical biology 1(4), 579-588.

Chen, X., Zhou, B., Li, M., Deng, Q., Wu, X., Le, X., Katsanis, E., 2007. CD4+ CD25+ FoxP3+ regulatory T cells suppress *Mycobacterium tuberculosis* immunity in patients with active disease. Clinical immunology 123(1), 50-59.

Chenthurpandy, P., Kalidass,. Mohan, V. R., 2009. Pharmacognostical Investigation of *Hiptage benghalensis* (L.) Kurz. (Malpighiaceae).Pharmacognosy Journal 1(2), 103-105.

Cowan, M. M., 1999. Plant products as antimicrobial agents. Clinical microbiology reviews 12(4), 564-582.

Ishida, S., Arai, M., Niikawa, H., Kobayashi, M., 2011. Inhibitory effect of cyclic trihydroxamate siderophore, desferrioxamine E, on the biofilm formation of *Mycobacterium* species. Biological and Pharmaceutical Bulletin 34(6), 917-920.

Kamatou, G. P. P., Viljoen, A. M., Gono-Bwalya, A. B., Van Zyl, R. L., Van Vuuren, S. F., Lourens, A. C. U., Steenkamp, P., 2005. The *in vitro* pharmacological activities and a chemical investigation of three South African *Salvia* species. Journal of ethnopharmacology 102(3), 382-390.

Lall, N., Henley-Smith, C.J., De Canha, M.N., Oosthuizen, C.B., Berrington, D., 2013. Viability reagent, presto blue, in comparison with ither available reagents, utilized in Cytotoxicity and antimicrobial assays. International Journal of Microbiology 2013, 1-5.

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Ma, Y., Stern, R. J., Scherman, M. S., Vissa, V. D., Yan, W., Jones, V. C., McNeil, M. R., 2001. Drug targeting *Mycobacterium tuberculosis* cell wall synthesis: genetics of dTDP-rhamnose synthetic enzymes and development of a microtiter plate-based screen for inhibitors of conversion of dTDP-glucose to dTDP-rhamnose. Antimicrobial agents and chemotherapy 45(5), 1407-1416.

Mishra, L. C., Singh, B. B., Dagenais, S., 2000. Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review. Alternative Medicine Review 5(4), 334-346.

Newton, S. M., Lau, C., Gurcha, S. S., Besra, G. S., Wright, C. W., 2002. The evaluation of forty-three plant species for in vitro antimycobacterial activities; isolation of active constituents from *Psoralea corylifolia* and *Sanguinaria canadensis*. Journal of ethnopharmacology 79(1), 57-67.

Niederweis, M., Danilchanka, O., Huff, J., Hoffmann, C., Engelhardt, H., 2010. Mycobacterial outer membranes: in search of proteins. Trends in microbiology 18(3), 109-116.

Ostrovsky, G., 2005.Online. Preventing biofilms in TB and leprosy. Available at: http://www.medgadget.com/2005/12/preventing_biof.html. (Accessed 15 February 2015).

Recht, J., Kolter, R., 2001. Glycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. Journal of bacteriology 183(19), 5718-5724.

Reyrat, J. M., Kahn, D., 2001. *M. smegmatis*. Trends in microbiology 9(10), 473-474.

Singh, N., Bhalla, M., de Jager, P., Gilca, M., 2011. An overview on ashwagandha: a Rasayana (rejuvenator) of Ayurveda. African Journal of Traditional, Complementary and Alternative Medicines 8(5S).

Udou, T., Ogawa, M., Mizuguchi, Y., 1982. Spheroplast formation of *Mycobacterium smegmatis* and morphological aspects of their reversion to the bacillary form. Journal of bacteriology 151(2) 1035-1039.

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Chapter 6

Overall conclusion & recommendations

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6. Conclusion

Tuberculosis is an infectious disease, mostly affecting lungs or abdominal area. The causative agent of TB is a bacterium called *Mycobacterium tuberculosis*. It is the second leading cause of death in the world. Natural sources have been considered as an alternative, as resistance of *M. tuberculosis* to commercially available drugs. Twenty South African medicinal plants were evaluated for their antituberculosis activity.

Twenty South African medicinal plant extracts were evaluated for *in vitro* antimycobacterial activity using the Microtiter Alamar Blue Assay and cytotoxicity with XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) assay. *Mycobacterium tuberculosis* H37Rv strain was used as the test organism. Cytotoxicity test was done on human macrophages (U937 cell line). Of all the 20 extracts 7 showed activity against *Mycobacterium* with minimum inhibitory concentration rangings from 125-31.25 µg/ml. *Ficus sur* had a selectivity index of 3 followed by *Salvia africana-lutea* and *Sphedamnocarpus pruriens* with a selectivity indexes of 2.

The plant extracts were further tested for their activity against Glutathione disulfide reductase (Human analogue) and mycothiol disulfide reductase (*Mycobacterium* analogue). The inhibitory activity of the plant extracts was determined using a DTNB-coupled glutathione/mycothiol disulfide reductase assays. *Typha minima* showed a potential inhibitory activity against Mtr with an effective concentration at which 50% activity is inhibited (EC_{50}) of 47.89±47.5µg/ml and had less inhibitory activity against Gtr with an EC_{50} of 813.5±3.21µg/ml.

The plant extracts were screened to evaluate whether they had antimicrobial and antibiofilm formation activity. Utilizing *Mycobacterium smegmatis* as the test organism, which is a genetic homologue to *Mycobacterium tuberculosis*. No significant antimicrobial activity was observed from the plant extracts. Qualitatively the plant extracts; *Sphedamnocarpus pruriens* (N), *Salvia africana lutea* (L), *Withania somnifera* (R) showed considerable antibiofilm activity and was validated by determining quantitavely. The effective concentration (EC_{50}) was determined and *Leonotis leonurus* L. (K), *Salvia africana lutea* (L), and *Sphedamnocarpus pruriens* (N) showed potential biofilm formation inhibition at concentration 45.55±0.2475µg/ml, 100±56.83µg/ml and 61.39±60.59µg/ml respectively.

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Two of the plant extracts; *Sphedamnocarpus pruriens* and *Salvia africana-lutea* showed good antimycobacterial and antibiofilm activity with *S. pruriens* being less toxic to U937 cell line. *Typhaminima* showed good Mycothiol reductase enzyme inhibition as it had less affinity for Gtr and higher affinity for Mtr. *Sphedamnocarpus pruriens* showed good overall activity when compared to all the plant extracts in this study.

An ideal plant or rather additive to tuberculosis treatment would show antimycobacterial activity, inhibition of Mycothiol reductase, with less toxicity to human cells and antibiofilm activity. In other words the plant extract would be active against planktonic and biofilm forming (extracellular) cells, intracellular activity with regards to inhibition of Mtr. Overall some of the plant extracts showed activity in both antimycobacterial and antibiofilm activities, while one plant only showed good activityagainst Mtr.

6.1 Recommendations for further studies

- Synergistic activity of the plant extracts which indicated good antimycobacterial activity
- Mechanism of action studies of the plant extracts with regards to mycothiol/glutathione disulfide reductase as potential drug targets must be done
- Isolation of pure compounds from *Sphedamnocarpus pruriens* , which indicated good overall activity

6.2 Manuscripts and presentations

6.2.1 Manuscripts under preparation

- **Gasa, N**., Oosthuizen, C.B., Lall, N., (2015). Antibiofilm activity of South African plant extracts against *Mycobacterium* spp. and their mechanism of action using Mycothiol reductase. *BMC Complementary and Alternative Medicine* (Under preparation)

6.2.2 Conference presentations

- The work represented in this dissertation will be presented at the **International Indigenous Plant Use Forum (IPUF) and Society for Economic Botany (SEB) conference,** Global vision on indigenous plants and economic botany, South Africa. 28 June-2 July 2015.

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Title: Antibiofilm activity of South African plant extracts against *Mycobacterium* spp. and their mechanism of action using Mycothiol reductase

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