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Antituberculosis activity of flavonoids from *Galenia africana* L. var. *africana*

BY

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DECLARATION

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DEDICATION

This thesis is dedicated to my late grandfather Abner F. Mativandlela and my family for being a positive motivating force in my life and supporting me through the trials and tribulations of life.

ABSTRACT

The recent increase in the incidence of tuberculosis (TB) with the emergence of multidrug-resistant (MDR) cases has lead to the search for new TB-drugs. *Mycobacterium tuberculosis* is a complex, resilient organism, and it is important to note that new drugs are required which can reduce TB's six month treatment time and can be effective against drug-resistant strains of mycobacteria. Plants contain numerous biological active compounds, many of which have been shown to have antimicrobial activity. The search for biologically active extracts based on traditional use of plants is relevant due to the appearance of microbial resistance to many antibiotics and the occurrence of fatal opportunistic infections. Ethanol extracts of seven selected ethnobotanically South African medicinal plants (*Artemisia afra, Dodonaea angustifolia, Drosera capensis, Galenia africana, Prunus africana, Syzygium cordatum* and *Ziziphus mucronata*) were investigated for their antimycobacterial activity against two *Mycobacterium* species.

The minimum inhibitory concentration (MIC) of ethanol extracts of *A. afra, Dodonaea angustifolia, Drosera capensis* and *G. africana* ranged from 0.781 to 6.25 mg/mL against a non-pathogenic strain of mycobacteria, '*M. smegmatis*'. *G. africana* showed the best activity, exhibiting an MIC of 0.781 mg/mL and a minimum bactericidal concentration (MBC) of 1.563 mg/mL against *M. smegmatis*. A drug sensitive strain of *M. tuberculosis* was found to be susceptible to the ethanol extracts of *Dodonaea angustifolia* and *G. africana*. (MICs 5.0 and 1.2 mg/mL respectively) when using the rapid radiometric-BACTEC method. The phytochemical analysis of *G. africana* led to the isolation and identification of three known compounds namely; (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-3,2',4'-trihydroxychalcone (not reported from natural sources) and (*E*)-2',4'-dihydroxychalcone. A novel chalcone '(*E*)-3,2',4'-trihydroxy-3'-methoxychalcone' was also isolated from the ethanol extract of *G. africana*. Isolation of (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-3,2',4'-trihydroxychalcone and *E*)-3,2',4'-trihydroxy-3'-methoxychalcone was reported for the first time from this plant. The MIC of novel compound against *M. tuberculosis* was found to be 50.0 µg/mL whereas (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-3,2',4'-trihydroxychalcone exhibited an MIC of 100.0 µg/mL.

During synergistic studies using (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone with the antituberculosis drug INH, it was found that the MICs of INH and the compounds were reduced sixteen and eight-fold respectively, resulting in a Fractional Inhibitory Concentration (FIC) of 0.1250 and 0.1875 respectively. The synergistic effect of two isolated compounds (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone) in *in vitro* studies also showed synergistic action, reducing their original MICs four-fold resulting in a FIC of 0.5. Since (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone from *G. africana* showed synergistic activity with INH, it is speculated that the compounds might have similar mechanism as that of INH. However, mechanistic studies on these compounds should be done in order to get an indication of the 'flavonoids and chalcones' interferences on mycolic acid synthesis, membrane synthesis and enzyme inhibition. Our investigation on the NADPH oxidase activity of (2*S*)-5,7,2'-trihydroxyflavanone with Mtr, found that this compound failed to exhibit any NADPH oxidase activity at 800 µM concentrations. Mtr is evidently not the target for the antimycobacterial activity of (2*S*)-5,7,2'-trihydroxyflavanone.

Fifty percent inhibitory concentration of the ethanol extract of *G. africana*, and the two purified compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone were found to be 120.0; 110.3 and 80.2 µg/mL respectively against the U937 cells. The MIC of the ethanol extract of *G. africana* in U937 macrophages infected with *M. tuberculosis* was found to be 50.0 µg/mL indicating the extract's intracellular antimycobacterial activity in real physiological conditions. The two purified compounds also showed good intracellular antimycobacterial activity. The MICs of (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone were found to be 100 and 50 µg/mL respectively. This study indicated that the intracellular activity of the ethanol extract is significant in macrophages. The activity might be due to *M. tuberculosis* being unable to avoid macrophage killing and its survival during phagocytosis, (including inhibition of phagosome-lysosome fusion, inhibition of the acidification of phagosomes, resistance to killing by reactive oxygen intermediates and modification of the lipid composition of the mycobacterial cell membrane, thereby altering its capacity to interact with immune or inflammatory cells).

It can be concluded that the traditional use of *G. africana* for TB has been scientifically validated to some extent. Isolated compounds and the ethanol extract of the plant warrant further investigation for their potential as antimycobacterial agents. Since synergistic activity of purified compounds with existing antituberculosis drug INH, was significant, it will be worthwhile evaluating the efficacy of purified compounds in combination with TB-drugs in pre-clinical studies.

List of Abbreviations

AIDS	Acquired immune deficiency syndrome
ATCC	American type culture collection
CFU	Colony forming units
CMI	Cell-mediated immunity
CNS	Central Nervous System
CR	Complement receptors
CSF	Cerebrospinal fluid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOT	Direct Observed Therapy
EMB	Ethambutol
FAS	Fatty acid synthase
FIC	Fractional inhibitory concentration
GI	Growth index
GPC	Gel permeation chromatography
GSK	GlakoSmithKline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
ICL	Isocitrate lyase
INH	Isoniazid
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl
LADH	Lipoamide dehydrogenase
LAM	Lipoarabinomannan
LD_{50}	Lethal dose, 50%
LJ	Lowënstein-Jensen
MBC	Minimal bactericidal concentration
MDR	Multidrug-resistant
MHC	Major histocompatibility complex

List of Abbreviations

MIC	Minimal inhibitory concentration
MR	Mannose receptors
MRC	Medical Research Council
MSH	Mycothiol
MSSM	Mycothiol disulfide
MTR	Mycothiol reductase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OD	Optical density
PAS	Para-aminosalicylic acid
PBS	Phosphate-buffered saline
POA	Pyrazinoic acid
PZA	Pyrazinamide
RIF	Rifampicin
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
STR	Streptomycin
TB	Tuberculosis
TH cell	T helper cell
TLC	Thin layer chromatography
TMP	Traditional medicinal practitioners
U937	Human leukemic monocyte lymphoma cell line
UV	Ultra violet light
WHO	World Health Organization
XDR	Extremely drug resistant

Table of contents

List of Tables

List of Figures

^{1}H and ^{13}C-NMR spectrums of isolated compounds from the ethanol extract of *G. africana*

(a) ^{13}C NMR of (2*S*)-5,7,2'-trihydroxyflavanone

(b) ^{1}H NMR of (2*S*)-5,7,2'-trihydroxyflavanone

(c) ^{13}C NMR of (*E*)-2',4'-dihydroxychalcone

(d) ^{1}H NMR of (*E*)-2',4'-dihydroxychalcone

(e) ^{13}C NMR of (*E*)-3,2',4'-trihydroxychalcone

(f) ^{1}H NMR of (*E*)-3,2',4'-trihydroxychalcone

(g) ^{13}C NMR of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

(h) ^{1}H NMR of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

Chemical structures of isolated compounds from the ethanol extract of *G. africana*

(a) (2*S*)-5,7,2'-trihydroxyflavanone

(b) (*E*)-2',4'-dihydroxychalcone

(c) (*E*)-3,2',4'-trihydroxychalcone

(d) (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

The HMBC spectrum of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

The comparative bactericidal effect of the ethanol extract, fractions and isolated compounds of *G. africana* against the drug-susceptibility strain of *M. tuberculosis*. Results illustrate the mean of the viable bacterial counts ± standard error in the treated vials as compared to the untreated control vials

Zones of inhibition of *M. smegmatis* on the TLC plates in a direct assay of (2*S*)-5,7,2'-trihydroxyflavanone isolated from the *G. africana* ethanol extract

TLC developed in hexane-ethyl acetate (8:2)

Chemical structure of mycothiol (MSH)

Structure of mycothiol disulfide reductase (Mtr)

Enzymatic reduction of naphthoquinone to semiquinone

CHAPTER 1

LITERATURE REVIEW: INTRODUCTION

1.1. Introduction

1.1.1. History of herbal medicine and plant derived drugs

Herbal medicine, sometimes referred to as herbalism or botanical medicine, is the use of plants for their therapeutic or medicinal value and has been used by all cultures throughout history (Duke, 2002). Plants produce and contain a variety of chemical substances that act upon the body. Herbalists use the leaves, flowers, stems, berries and roots of plants to prevent, relieve, and treat illnesses (Wijesekera, 1991). About 25% of the prescription drugs dispensed in the United States contain, at least, one active ingredient derived from plant material, some are made from plant extracts and others are synthesized to mimic a natural plant compound (Balick, 1990). A number of herbal plants and their compounds have been used, and have served as models for modern medicine (Farnsworth, 1984). Many drugs listed as conventional medications were originally derived from plants. Salicylic acid, a precursor of aspirin, was originally derived from 'white willow bark' and the 'meadowsweet plant' (Gurib-Fakim, 2006). Well known examples of plant derived drugs include the antimalarial 'quinine', extracted from the bark of the 'Cinchona' species. 'Vincristine', which is being used to treat certain types of cancer, comes from the 'Madagascar periwinkle' (*Catharanthis roseus*). In 1819, the isolation of the analgesic morphine, codeine and paregoric laid down the foundation for the purification of pharmacologically active compounds for the treatment of diarrhoea (Potterat *et al*., 2008). 'Laudanum', a tincture of the 'opium poppy', was the favoured tranquilliser in Victorian times. Even today, morphine, the most important alkaloid of the opium poppy, remains the standard against which new synthetic analgesic drugs are measured (Phillipson, 2001; De Smet, 1997).

1.1.2. Use of medicinal plants

The use of plants as the source of remedies for the treatment of many diseases dates back to prehistory and people of all continents have this old tradition. Plants continue to be a major source of medicines, as they have been throughout human history (van Wyk *et al*., 1997). Up to 80% of the world population, South Africa population use medicinal plants as remedies. Plant species serve as a rich source of many novel biologically active compounds, although very few have been thoroughly investigated for their medicinal properties (Heinrich and Gibbons, 2001). Apart of the 30% and 40% used in today's conventional drugs, other plants are used as herbal supplements, botanicals and teas (Kirby, 1996; Hostettmann and Marston, 2002). The World Health Organization (WHO) estimates that 4 billion people, or 80% of the world population, presently use herbal medicine for some aspect of primary health care. Herbal medicine is a major component in all indigenous peoples' traditional medicine and is a common element in ayurvedic, homeopathic, naturopathic, traditional Oriental and Native American Indian medicines. WHO notes that of the 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures (Shulz *et al*., 2001). The uses of some medicinal plants vary a lot according to regional and cultural aspects. Their use is often associated with witchcraft and superstition, because people do not have the scientific insight to explain or predict the curative action of plants. One example of such an irrational concept is the Doctrine of Signatures (elements of which are found in many of the healing cultures of the world), which is based on the assumption that the appearance of plants may give clues to their medicinal properties (van Wyk and Wink, 2004).

Africa has a long history of people-plant interaction. The continent is characterised by rich ethnic and biotic mosaics that represent 13% of the earth's human population and has one of the largest continental floras of which estimates range from between 50 000 and 70 000 plant taxa (Nigro *et al*., 2004; Klopper *et al*., 2002; Smith and van Wyk, 2002). The African flora is remarkable not only for its diversity but its distinctiveness: as many as 88% of its species are endemic. High levels of endemism

indicate that many of the continent's plant resources are uniquely African and are used in agriculture, horticulture, medicine, forestry, etc (Nigro *et a*l., 2004; Davis *et al*., 1994). African traditional medicine is one of the oldest and the most diverse of all medicinal systems. More than 70% of the population refers to traditional healers concerning health issues. The healer typically diagnoses and treats the psychological basis of an illness before prescribing medicines to treat the symptoms (van Wyk and Wink, 2004). Famous African medicinal plants include *Acacia senegal* (gum Arabic), *Agathosma betulina* (buchu), *Aloe ferox* (Cape aloes), *Artemisia afra* (African wormwood), *Boswellia sacra* (frankincense), *Catha edulis* (khat), *Commiphora myrrha* (myrrh), *Hibiscus sabdariffa* (hibiscus), *Hypoxis hemerocallidea* (African potato) and *Prunus africana* (red stinkwood) (van Wyk and Wink, 2004). The Khoi-San people of southern Africa, nowadays considered to be the most ancient of all cultures, have a remarkable *Materia medica* (medicinal plants and other materials) which typically includes general tonics, fever remedies, sedatives, laxatives and numerous wound healing plants.

There are an estimated 200 000 indigenous traditional healers in South Africa, and more than 60% of South Africans consult these healers, usually in addition to using modern biomedical services and commonly used medicinal plants (Table 1.1). Traditional healers in South Africa are most commonly known as "inyanga" (Zulu) and "tin'anga" (Xitsonga) (van Wyk *et al*., 1997). Traditional medicines are well recognised and different communities use a wide variety of plants to treat gastrointestinal disorders such as diarrhoea, tuberculosis, malaria, sexual transmitted diseases and other infections which are particularly prevalent in rural area (McGraw *et al*., 2000; Yelne *et al*., 2001). Although South Africa contains about 10% of the earth's plant diversity, relatively little work has been done on medicinal plants from this region. South Africa's rich heritage of indigenous medicines coupled with biodiversity, form extremely valuable resources.

Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or synergy to improve health. A single plant may contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce

Table 1.1. Commonly used medicinal plants (Yelne *et al*., 2000)

Achillea millefolium (yarrow)	*Chicorium intybus* (chicory)	*Leonurus cardiaca* (motherwoth)	*Pimpinella anisum* (anise)
Acourtia runcinata (peonia)	*Cinnamomum camphora* (camphor)	*Lavandula angustiolia* (lavender)	*Pinus sylvestris* (scots pine)
Aloe vera (aloe)	*Cinnamomum zeylanicum* (cinnamon)	*Majorana hortensis* (marjoram)	*Pogostemon cablin* (patchoul)
Allium sativum (garlic)	*Citrus aurantium* (bitter orange)	*Malva parviflora* (malva)	*Rauvolfia serpentina* (rauvolfia)
Amarylis belladonna (belladonna)	*Coriadrum sativum* (coriander)	*Maranta arundinacea* (arrowroot)	*Rhamnus cathartica* (buckthorn)
Angelica antropurpurea (angelica)	*Elettaria cardamomum* (cardamon)	*Marrubiun vulgare* (horehound)	*Rheum rhabarbarum* (rhubarb)
Anethum graveolens (dill)	*Eucalyptus globus* (eucalyptus)	*Melissa officinalis* (balm)	*Rubus fruticosus* (blackberry)
Annona squamosa (apple)	*Eugenia caryophyllata* (clove)	*Mentha arvensis* (mint)	*Ruta graveolens* (rue)
Apium graveolens (celery)	*Foeniculum vulgare* (fennel)	*Monarda fistulosa* (bergamot)	*Sabal palmetto* (palmetto)
Arnica alpina (arnica)	*Gentiana lutea* (gentiana)	*Ocimum basilicum* (basil)	*Sesamum indicum* (sesame)
Artemisia spp. (artemisia)	*Gingko biloba* (gingko)	*Olea europaea* (olive)	*Turnera diffusa* (damiana)
Calendula officinalis (calendula)	*Grindelia robusta* (gumplant)	*Origanum vulgare* (oregano)	*Taraxacum officinale* (dandelion)
Cammiphora myrrha (myrrh)	*Hypericum perforatum* (St John's wort)	*Panax quinquefolia* (ginseng)	*Urtica* spp. (nettle)

swelling and pain, phenolic compounds that act as antioxidants and venotonics, antibacterial and antifungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste product and toxins and alkaloids that enhance mood and give a sense of well-being (van Wyk and Wink, 2004). The importance of plants lies not only on their chemotherapeutic effect, but also in their role as a source of model compounds for drug development. In addition to plant constituents being used directly as therapeutic agents, they can be utilized as starting material or templates for drug synthesis (Eloff, 1998). In addition to active ingredients, the plant's bioflavanoids and other substances are important in supporting its medicinal properties. These elements also provide an important natural safeguard. Isolated or synthesized active compounds can become toxic in relatively small doses; unlike a whole plant which reaches a toxic level only when taken in large quantities (Eloff, 1998).

Medicinal plants are also important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). Major pharmaceutical companies are currently conducting extensive research on plant materials, gathered from forests and other habitats, for their potential medicinal value. Rather than using a whole plant, scientists identify, isolate, extract, and synthesize individual components, thus capturing the active compounds. There are over 750,000 plant species on earth, but relatively speaking, only a very few of the healing plants have been studied scientifically. Because modern pharmacology looks for one active ingredient and seeks to isolate it to the exclusion of all the others, most of the research that is done on plants continues to focus on identifying and isolating active ingredients, rather than studying the medicinal properties of the whole plants. Herbalists, however, consider that the power of a plant lies in the interaction of all its ingredients. Plants used as medicines offer synergistic interactions between ingredients both known and unknown (Mabogo, 1990). Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in industrialized countries are derived directly or indirectly from plants (Newman *et al*., 2003).

1.1.3. Selection of medicinal plants and plant-parts used in South Africa

In order to know how to select the most appropriate medicinal plants, the therapeutic specifics must be understood. Choice of dosages must be based on the needs of the patient, the method of preparation which is affected by the chemistry of the plant constituents considered to be responsible for the therapeutic effects and the administration of plant-based drugs are very important. The part of the plant used varies among species, traditional healers and also depends on the nature and state of the disease (Mabogo, 1990). Different parts of a plant (leaves, roots, bark, fruit and seeds) often contain quite different active ingredients, so that one part may be toxic and another one quite harmless (van Wyk and Wink, 2004). The VhaVendas in the Venda region of Lipompo province of South Africa most often prepare a decoction of the plant part in soft porridge (Arnold and Gulumian, 1984). Babies for example, are generally given a soft porridge called 'tshiunza', made from flour mixed with a number of medicinal plants. The immune system of the child is expected to be strengthened by this preparation. Other forms of dosage employed in traditional preparation of medicinal plants include: maceration, juice, syrup, tincture, medicinal ointments, infusion, decoction, digestion and percolation. There are also medicinal products that are mixtures and contain two or more herbs that act individually, additively or even synergistically to restore or maintain health (van Wyk and Wink, 2004).

Traditional healers use medicinal plants for a variety of illness such as chest pains, tuberculosis (TB), malaria, diarrhoea, appetite suppressant, arthritis, asthma, etc (Cragg and Newman, 2005). A few of these plants have been scientifically investigated and valuable products (either in the form of herbal supplements or novel drugs in the form of isolated compounds) are currently going through clinical trials. Examples are: *Hoodia gordonii* which grows naturally in South Africa and Namibia, is used as appetite suppressant. *Catharanthus roseus*, endemic to Madagascar, is used for treatment of diabetes and menorrhagia. *Combretum caffrum*, indigenous to South Africa, is used for cancer. *Pelargonium sidoides*, widely distributed in South Africa, is used for respiratory problems including bronchitis and TB. Examples of plants that

are being used to treat tuberculosis in South Africa are: *Cryptocarya latifolia*, *Chenopodium ambrosioides*, *Euclea natalensis*, *Ekebergia capensis*, *Helichrysum melanacme*, *Nidorella anomala*, *Polygala myrtifolia* and *Thymus vulgaris* (Lall and Meyer, 1999).

1.2. Medicinal plants with antimycobacterial activity

Over the past decade there has been a proliferation of literature on the antibacterial, antituberculosis, antifungal and antiviral properties of plant extracts. Screening plant extracts for antimycobacterial activity is usually carried out using mycobacteria cultured in various types of broth and agar based media (Newton *et al*., 2000). There are several reports on *in vitro* inhibition of mycobacterium species by medicinal plants and the bioassay-guided research for antimycobacterial properties from plants has shown signs of success. Major review articles have appeared on antimycobacterial natural products in the last eight years. Although no marketable products for the treatment of TB have been isolated from plants, some lead compounds have been identified (Cantrell *et al*., 1999). Cantrell *et al*., (2001) isolated norditerpenoid, '12 demethylmulticauline', from the roots of *Salvia multicaulis*, which was more active than the first line TB drugs ethambutol (EMB) and nearly as active as rifampicin (RIF) *in vitro*. Mitscher and Baker discussed plant-derived compounds (berberine, lichoisoflavone, erygibisoflavone, phaseollidin, erythrabyssin II and tryptanthrin) as potential antituberculosis agents (Mitscher and Baker, 1998). Newton *et al*., 2000 reviewed plant-derived antimycobacterial natural products, describing the activity of extracts and compounds from 123 plants species (Newton *et al*., 2000).

Reports of 88 naturally occurring compounds and synthetic analogues from plants, fungi and marine organisms, that demonstrated significant activity in the *in vitro* bioassays against *M. tuberculosis* and other mycobacterial species, have been described (Okunade *et al*., 2004). Recent developments in mycobacteriology and innovative natural products chemistry tools and their potential to impact on the early steps of the TB drug discovery process have been reviewed (Pauli *et al*., 2005).

Gautam *et al*., 2007 described 70% (255 of 365) of Indian medicinal plant species, from a wide range of families, which have shown antimycobacterial activity. Interestingly, when tested preliminary in the *in vitro* screening, 149 species have shown positive ethnomedicinal uses in correlation with the traditional knowledge for TB or related diseases (Gautam *et al*., 2007).

Ten of the 408 ethanolic extracts of plants such as *Actaea spicata*, *Angustura vera*, *Cinnamonium camphora*, *Piper cubeba*, *Guauacum officinale*, *Ipomea purga*, *Rhamnus cathartica* inhibited growth of *M. tuberculosis* H37Rv at dilutions of 1 in 160 to 1280 and a high proportion of the other extracts inhibited growth at lower dilutions (Grange and Dawey, 1990). It was found that *M. tuberculosis* was also sensitive towards *Pentas longifolia*, *Tetradenia riparia* and *Bidens pilosa*, medicinal plants used in Rwanda. The active compound isolated from the leaves of *T. riparia* was tested against *M. tuberculosis* which showed activity at 100 µg/mL (van Puyvelde *et al*., 1994). *Hydrocotyle asiaticum* inhibited growth of *M. tuberculosis* at a dilution of 1:20 (Grange and Dawey, 1990). Organic extracts of *Helichrysum crispum* inhibited the growth of *M. tuberculosis* (Salie *et al*., 1996). Lall and Meyer (2001) demonstrated the inhibition of drug-sensitive and drug-resistant strains of *M. tuberculosis* by diospyrin isolated from the roots of *Euclea natalensis* AD. The phytochemical and biological studies of *E. natalensis* and isolated compounds from this plant indicated *in vitro* activity against *M. tuberculosis*. In structure-activity related studies against *M. tuberculosis* different research groups have found activity using a variety of natural products with no definite trend towards a specific group of compounds (Houghton *et al*., 1999).

In South Africa, it has been reported that people smoke dried flower and seed of *Helichrysum krausii* in a pipe for the relief of coughs and as a remedy for pulmonary TB (Watt and Breyer- Brandwijk, 1962). Chelerythrine isolated from methanolic root extracts of *Sanguinaria canadensis* was found to be the most active isolated compound against *M. smegmatis* at 29.0 µg /mL (Newton *et al*., 2002).

A number of plants have been cited in the literature as being used for medication against various bacterial and viral infections or as containing biologically active compounds. Research conducted by Noristan, Pretoria, suggests that from a total number of about 300 plants screened, at least 31% show marked analgesic, anti-inflammatory and anti-infective properties (Theunis *et al*., 1992).

Plants have been endowed with therapeutic virtues both in legend and in scientific literature and are being used in treating various ailments such as coughs, colds, other pathogenic bacterial and viral infections. The use of antimicrobials from the natural vegetation has a great impact in human health care in undeveloped countries. Herbal medicine has been used for centuries in rural areas by local healers and has been improved in industrialized countries. A number of substances used in modern medicine for the treatment of serious diseases have originated from research on medicinal plants (Theunis *et al*., 1992). In the present study plants used for TB-related symptoms were scientifically investigated for antimycobacterial activity.

1.3. Scope of thesis

1.3.1. Antimycobacterial activity of selected medicinal plants

In this study, the antimycobacterial activity of selected South African medicinal plants, that have been used in the treatment of TB symptoms, was investigated.

Ethanol crude extracts were screened against non-pathogenic strain of mycobacteria, '*M. smegmatis*'and a pathogenic strain, '*M. tuberculosis*'.

1.3.2. Cytotoxicity of the crude extracts

Cytotoxicity evaluation of the plant extracts using African monkey kidney vero cells were carried out with the intention of choosing a plant for the isolation of the active compounds with anti-TB activity and low toxicity.

1.3.3. Bioassay guided fractionation of *Galenia africana*

It was found that *G. africana* possessed high antimycobacterial activity and has less toxicity than the other plants investigated. Our objective was to isolate the active compound(s) and evaluate the minimal inhibitory concentration (MIC) against *M. tuberculosis*. Through the bioassay guided fractionation of the ethanol extract of *G. africana*, four compounds were isolated and identified.

1.3.4. Synergistic activity of the isolated compounds

Synergistic inhibitory activity of the isolated compounds was also investigated against *M. tuberculosis* using the radiometric BACTEC method.

1.3.5. Intracellular antimycobacterial activity of selected samples

It has been reported that *M. tuberculosis* may survive in macrophages by various mechanisms and that anti-TB drugs effective on macrophages are therefore needed for the treatment of TB. It was therefore decided to investigate the cytotoxicity of the ethanol extract of *G. africana*, the isolated compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone on U937 cell lines and their intracellular activities against U937 cells which were infected with *M. tuberculosis*.

1.3.6. Mechanism of action

Mycothiol (MSH) or 1d-*myo*-inosityl 2-(*N*-acetyl-l-cysteinyl)amido-2-deoxy-*α*-d-glucopyranoside, is an unusual conjugate of *N*-acetylcysteine (AcCys) with 1d-*myo*-inosityl 2-acetamido-2-deoxy-α-d-glucopyranoside (GlcN-Ins), and is the major low-molecular-mass thiol in mycobacteria. *M. tuberculosis* lacks glutathione, but instead maintains millimolar concentrations of the structurally distinct low molecular weight thiol MSH. MSH has antioxidant activity as well as the ability to detoxify a variety of toxic compounds. Because of these activities, MSH is a candidate for protecting *M. tuberculosis* from inactivation by the host during infections as well as for resisting

antituberculosis drugs. In order to define the protective role of MSH for *M. tuberculosis*, we investigated the inhibitory activity of selected antituberculosis extracts / compounds against mycothiol reductase, an enzyme responsible for the production of mycothiol.

1.4. Structure of thesis

The contents of each chapter are as follows:

Chapter 1: Literature on the potential of medicinal plants for antimycobacterial activity for various ailments.

Chapter 2: The epidemiology, prevention and treatment of TB and the anti-TB drugs which are in clinical trials.

Chapter 3: The selection, description and phytochemical constituents of selected South African medicinal plant species.

Chapter 4: The antituberculosis activity of selected South African medicinal plants against *M. smegmatis* and *M. tuberculosis*.

Chapter 5: Cytotoxic activity of selected South African medicinal plants.

Chapter 6: The bioassay-guided fractionation of the ethanol extract of *G. africana* and the identification of the bioactive compounds.

Chapter 7: The antimycobacterial activity of the fractions and isolated compounds from the ethanol extract of *G. africana*.

Chapter 8: The synergistic effect, cytotoxicity and intracellular antimycobacterial activity of the ethanol extract and isolated compounds from *G. africana*. Mechanism

of action of selected candidates is also discussed in this chapter.

Chapter 9: General discussion, conclusion and summary of the entire research and the importance of medicinal plants as traditional medicines.

Chapter 10: Summary.

Chapter 11: References.

Chapter 12: Acknowledgements.

Chapter 13: Appendices - Publications

CHAPTER 2
EPIDEMIOLOGY, PREVENTION AND TREATMENT OF TUBERCULOSIS

2.1. Introduction

2.1.1. History of tuberculosis

Tuberculosis (TB) is a disease known since antiquity and evidence of spinal TB in the form of fossil bones dates back to around 8000 BC (Ayyazian, 1993; Basel, 1998). TB occurred as an endemic disease among animals long before it affected humans (Steele and Ranney, 1958). The first confirmed instance of TB in humans was noted in the deformities of the skeletal and muscular remains of the Egyptian mummies of around 2400 BC (Haas, 1996). However, it could not be determined whether the disease was due to *M. bovis* or *M. tuberculosis*. In the 1700s and early 1800s, TB prevalence peaked in Western Europe and the United States and was undoubtedly the largest cause of death. Hundred to 200 years later, it had spread in full force to Eastern Europe, Asia, Africa and South America (Bloom and Murray, 1992).

2.1.2. Mycobacterium species

The genus *Mycobacterium* (order Actinomycetales, family Mycobacteriaceae) consists of about 50 acid-fast, aerobic, non-motile and non-spore-forming bacterial species. Most of these species are environmental saprophytes, existing in various substrate including soil, water, plants, mammals and birds. The genus is divided into the fast-growing and the slow-growing species. The fast-growing species are usually not pathogenic but some may cause opportunistic infections in animals and humans (Grange and Yates, 1986). The pathogenic species are obligate parasites and cause TB in humans and animals (McGaw *et al*., 2008).

2.1.2.1. *Mycobacterium tuberculosis*

M. tuberculosis was described on the 24th of March 1882 by Robert Koch, who in 1905 received the Novel Prize in physiology or medicine for this discovery (Newton *et al*., 2000). *M. tuberculosis* is also known as Koch's bacillus and it is a member of the "tuberculosis complex", a group of closely related mycobacterial pathogens, which include *M. bovis* (which infects cattle and may also infect humans), *M. microti*, *M. africanum* (which causes TB in West Africa), *M. avium*, *M. intracellulare*, *M. leprae* (causes leprosy in man), *M. lepraemurium* infection in rats and cats and *M. scrofulaceum* (causing opportunistic infectious disease in patients with AIDS, Hourne, 1996).

M. tuberculosis the causative agent of TB in humans is a fairly large nonmotile rod-shaped bacterium distantly related to the actinomycetes. The rods are 2-4 um in length and 0.2-0.5 um in width (Figure 2.1). The major components of the cell wall structure of the bacteria consist of peptidoglycan and lipids. Mycolic acids, which are α-branched lipids in cell walls, make up 50% of the dry weight of the mycobacterium cell envelope and are very strong hydrophobic molecules that form a lipid shell around the organism (Goren, 1990). Cord factor is a glycolipid (trehalose dimycolate) found in the cell wall that induces replication *in vitro*, resulting in serpentine cords of organisms. The role of the cord factor in the pathogenesis of TB is still under investigation, however, it is thought to be important because it inhibits and induces secretion of TNF-alpha by macrophages (Brennan, 1998).

Figure 2.1. Rods of *M. tuberculosis*, magnification x 6.250 (based on a 35 mm slide *image* of 24 mm in the narrow dimension (Courtesy: SEM/ 97229A)

M. tuberculosis is not classified as either a Gram-negative or Gram-positive bacteria because it does not have the biochemical characteristics of either (Camus *et al.*, 2002). If a Gram stain is performed on *M. tuberculosis*, it stains very weakly Gram-positive or not at all. *Mycobacterium* species, along with members of a related genus *Norcardia*, are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. One acid-fast staining method for *M. tuberculosis* is staining with carbon-fuchsin (a pink dye) and decolourising with acid alcohol. The smear is counterstained with methylene blue or certain other dyes on different media such as Lowenstein-Jensen (Figure 2.2; Fadda and Roe, 1984).

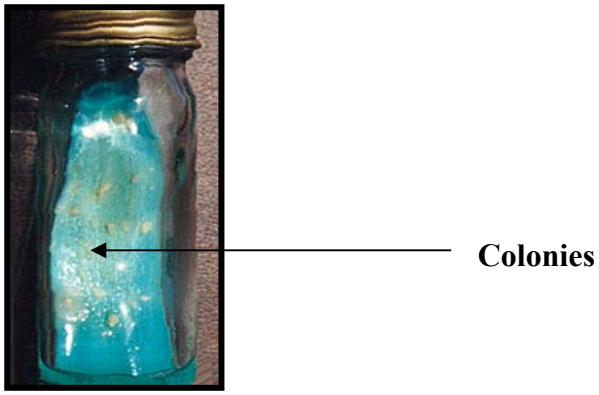


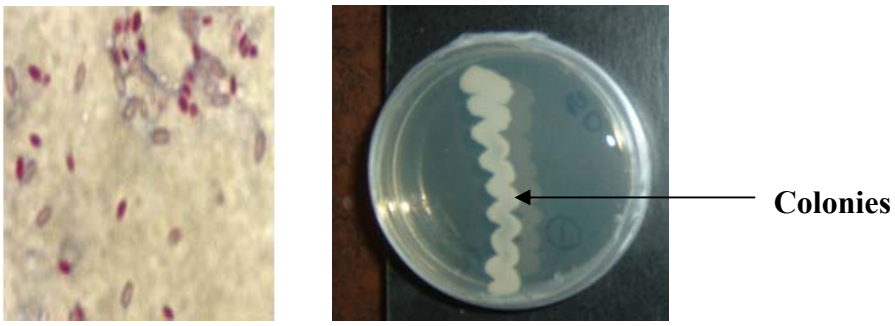
Figure 2.2. Colonies of *M. tuberculosis* on Lowenstein-Jensen medium

M. tuberculosis is a slow-growing bacillus which is transmitted primarily by the respiratory route. Infection with *M. tuberculosis* occurs by inhalation of small (1 – 10 microns) droplets containing only a few live tubercle bacilli. The primary focus of infection is usually the middle or lower zones of the lung. The bacilli are readily taken up by lung macrophages but can survive and grow to form the primary focus of infection and from there, enter the local lymphatic system and then move throughout the body via the blood and lymphatic system. This stage (the local lymphatic system), of disease is usually clinically silent or associated with mild fever and in most cases immunity develops within a few weeks and the patient becomes tuberculin positive (Girling, 1989). A key characteristic of *M. tuberculosis* infection is that the bacterium multiplies intracellularly, primarily in macrophages and in this way evading many host defence mechanisms (Banki *et al*., 1999; Velasco-Velazquez *et al*., 2003).

M. tuberculosis infects about 32% of the world's population. Every year, approximately 8 million of these infected people develop active tuberculosis and almost 2 million of these will die from the disease (WHO, 2006).

2.1.2.2. *Mycobacterium smegmatis*

M. smegmatis is a gram positive bacteria, belonging to the family Mycobacteriaceae and the genus *Mycobacterium* (Megehee and Lundrigan, 2007). The bacterial species is found in the soil, water and plants. It is acid-fast staining (Figure 2.3a) that shares many features with the pathogenic *M. tuberculosis*. It was first reported in November 1884 by Lustgarten who found a bacillus with the staining appearance of tubercle bacilli in syphilitic chancres. *M. smegmatis* is generally considered a fast growing non-pathogenic microorganism that can be cultured in any laboratory and the culture smear is grown on media such as Middlebrook 7H11 agar (Figure 2.3b), however, in some cases it can cause disease, mainly in animals (Niederweis *et al*., 1999). *M. smegmatis* possess a limited degree of similarity to *M. tuberculosis* with regard to drug susceptibility (Gautam *et al*., 2007).



(a) (b)

Figure 2.3. ***M. smegmatis***

(a) Rods of *M. smegmatis*: acid-fast stained red (due to carbon fuchsin dye), magnification x 1000, taken in a general microbiology lab (slide *image* of 21 mm in the narrow dimension (Courtesy: SEM/ 97229A)

(b) Colonies of *M. smegmatis* on Middlebrook 7H11 agar medium

2.1.3. Epidemiology

In the late 1980s, TB began re-emerging and now globally, kills more than 2 million people each year. It is thought that as many as 2 billion people have been exposed to the TB bacillus and are therefore at risk of developing the active disease (Gutierrez-Lugo *et al*., 2005). According to WHO (2001), TB is known to be the largest cause of death of the human species. There has been a resurgence of the disease over the last two decades with currently eight million new cases and about 200,000 deaths annually. It is estimated that between 2000 and 2020, nearly one billion people will become infected, 200 million will acquire the disease and 35 million will die from TB (WHO, 2000), in contrast to the 1.6 million deaths from TB in 2005. Both the highest number of deaths and the highest mortality rate are in the Africa region. The two essential factors for the rapid spread of TB are; crowded living conditions, which favour airborne transmission and a population with little natural resistance. TB in populations can be attributed to three distinct factors:

- Infection of an individual in the community with tubercle bacilli within a given time period.
- Development of the disease shortly after such infection.
- The disease developing long after the original infection, owing to the reactivation of latent bacilli (Raviglone *et al*., 1995; Bloom, 1994).

Today, TB is the leading cause of death worldwide from a single human pathogen, claiming more lives than diseases such as Human Immunodeficiency Virus / Acquired Immune deficiency Syndrome (HIV/AIDS), malaria, diarrhoea, leprosy and all the other tropical diseases combined (Zumla and Grange, 1998). The pandemic of HIV/AIDS infection and the evidence of an association with TB, have caused marked increases in the incidence of the disease in some countries (Bloom, 1994). Because of its ability to destroy the immune system, HIV has emerged as the most significant risk factor for progression of dormant TB infection to clinical disease (Selwyn *et al*., 1989). The Global Programme on AIDS of the WHO estimated that in 1992 at least 13 million adults and 1 million children had been infected with HIV worldwide

(WHO, 1999). The impact of HIV/AIDS infection on the TB situation is greatest in those populations where the prevalence of TB infection in young adults is very high (Bloom, 1994).

The number of cases worldwide is now increasing rapidly due to multi-drug resistant strains of *M. tuberculosis* as a result of patient non-compliance and also due to an increase in patients with HIV/AIDS (Collins, 1998; Zumla and Grange, 1998). About 450 000 multi-drug resistant tuberculosis (MDR-TB) cases are estimated to occur every year, the highest rates are in countries of the former Soviet Union and China (WHO, 2006). There are a number of countries that have made remarkable progress in expanding population coverage with cure rates, whereas South Africa battles with more than 188 000 new TB cases per year (Bloom, 2002). South Africa is burdened by one of the worst TB epidemics in the world, with the disease rates more than double of those observed in other developing countries and up to 60 times higher than those currently seen in developed countries (Bapela, 2005).

The TB problem in South Africa is largely as a result of historical negligence and poor management systems, compounded by the legacy of fragmented health services. In South Africa, a high proportion of the population lives under poor condition and this may lead to the disease becoming uncontrollable (Fourie and Weyer, 2000). South Africa has by far the worst TB prevalence rate in the world, with 998 South Africans out of every 100 000, living with TB (WHO, 2008). More than 280 000 cases of TB were reported in different South African provinces in 2006, which is an increase of 98% since 2001 when just over 120 000 TB cases were reported. In 2006, there were more than 131 000 new infectious cases and a 57% increase, since 2001 of spreading the disease to others (Figure 2.4., Tuberculosis Fact Sheet, 2007). The breakdown of TB patients reported from 2001 - 2006 in South Africa increased yearly, and the primary aim of the South African National TB Control Programme is to cure all new smear positive patients first time around (Table 2.1). The South African Medical Research Council (MRC) estimated 273 365 new cases of TB in the year 2000, of which 113 945 were infectious and 46,7% were HIV related.

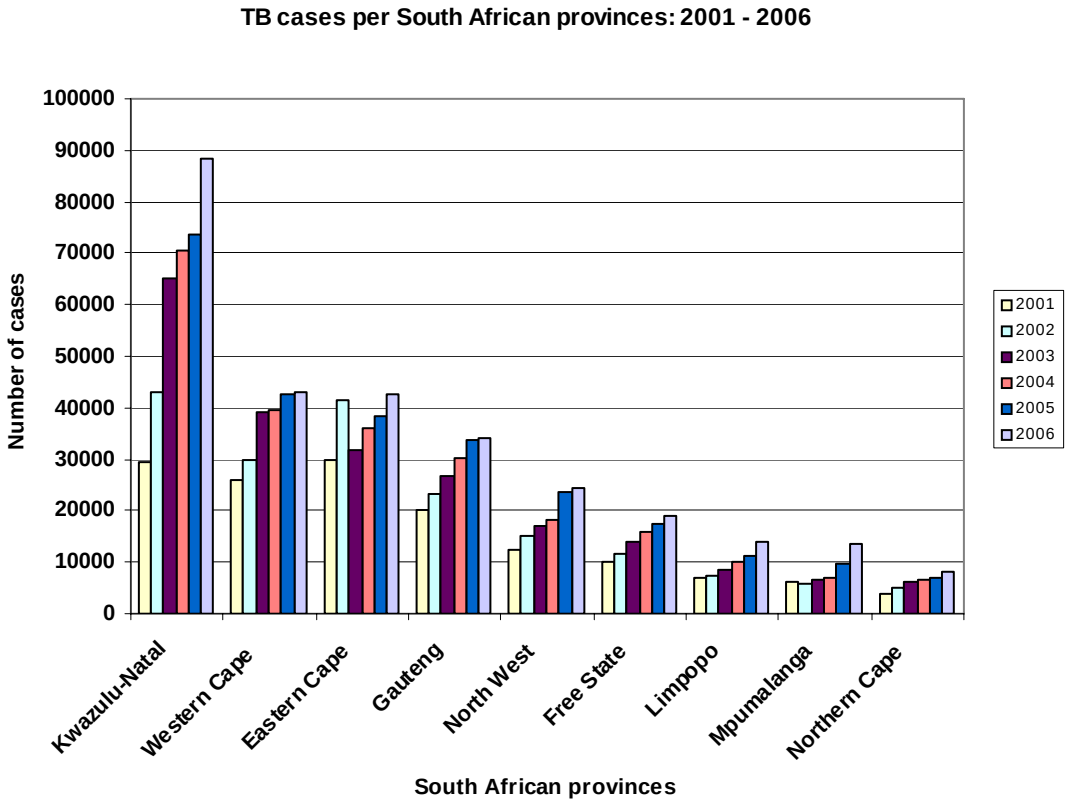


Figure 2.4. The increase of reported TB cases per South African provinces from 2001 - 2006 (Tuberculosis Fact Sheet, DoH -RSA, 2007)

Table 2.1. Breakdown of TB patients reported from 2001 - 2006 in South Africa (Tuberculosis Fact Sheet, DoH -RSA, 2007)

Cases	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**
TB	188 695	224 420	255 422	279 260	302 467	342 315
Pulmonary TB	144 910	182 583	215 154	234 213	257 604	287 440
New smear positive pulmonary TB	83 808	98 800	116 337	117 971	125 460	131 619
Re-treatment pulmonary TB	20 686	25 091	30 331	32 882	37 541	40 736

TB is not distributed evenly, throughout South Africa and the rates vary considerable among the nine provinces (Table 2.2). Current strategies for the control of TB centres around treatment with multi-drug regimes based on the very effective combination of isoniazid (INH., Figure 2.5) and rifampicin (RIF., Figure 2.6). In endemic areas, the

diagnosis and treatment of smear positive patients are emphasized in order to interrupt the spread of the disease within the community. Obstacles to the success of this strategy are the difficulties of early diagnosis and operational problems associated with delivery of a treatment that involves administration of multiple drugs over a period of at least six months (Young and Duncan, 1995).

Table 2.2. Occurrence of TB in different provinces of South Africa during the year 2000: 600 total cases per 1000 000 population; 250 smear-positive cases per 100 000 population (Fourie and Weyer, 2000)

Name of Province	Total TB cases	Proportion HIV+
Eastern Cape	56 495	40.0%
Gauteng	45 498	44.8%
Free State	14 654	51.7%
Kwazulu Natal	65 695	64.6%
Limpopo	23 338	36.3%
Mpumalanga	15 657	59.1%
Northern Cape	4 649	33.2%
North West	15 549	45.5%
Western Cape	34 211	31.6%
South Africa	**273 365**	**46.7%**

2.1.4. Transmission of tuberculosis

The principal risk method of being infected with TB is by inhaling contaminated air containing microbes that cause this disease (Rom and Garay, 1996). TB microbes can be present in sufficient concentrations in the air to cause infections and the disease. Once in air, water evaporates from the surface of a particle, decreasing its size and concentrating its contents of microbes. These particle forms a droplet nuclei in which evaporation continues until the vapour pressure of the droplet equals to the atmospheric pressure. The droplet nuclei are very stable, settle very slowly and remain suspended in the air for very long periods. Droplet nuclei are produced when a

patient with active pulmonary or laryngeal TB coughs, speaks, sneezes or sings. Coughing can produce 3000 infectious droplet nuclei, talking for 5 minutes an equal number and sneezing can produce over a million particles with a diameter of less than 100nm (Bloom, 1994). When inhaled, droplet nuclei usually travel through the airway until they reach the alveoli. Larger particles that are deposited on the way are removed through normal mechanism of airway clearance (Dannenberg, 1989).

2.1.5. Immunology of tuberculosis

TB is a prototype infection that requires control by the cellular immune response. In the first few weeks the host has almost no immune defence against infection by the bacteria causing TB. Small inhalation inocula multiply freely in the alveolar space or within alveolar macrophages. Unrestrained bacterial multiplication proceeds until the development of tissue hypersensitivity and cellular immunity intervene. The organism causing TB adheres to alveolar macrophages via multiple complements and might be destroyed in the phagosome (Beyers, 1999). The intracellular mechanisms for killing or inhibiting the growth of the bacteria in alveolar macrophages include the production of nitric oxide and reactive oxygen intermediates. Alveolar macrophages can also participate, in a broader context of cellular immunity, through the process of antigen presentation and recruitment of T-lymphocytes, which are the white blood cells produced in the bone marrow but which mature in the thymus. These cells are important in the body's defence against certain bacteria and fungi (Beyers, 1999).

Macrophages which are antigens are processed in phagosomes via Major Histocompatibility Complex (MHC) class II molecules to CD4 T-lymphocytes which are the major effector cells in cell-mediated immunity. The antigens bind to T-cell receptors on the surface of the T-lymphocytes. These CD4 T-lymphocytes tend to polarize into either Th1 cells (these are essential in controlling intracellular pathogens), producing predominantly interferon gamma (IFN-γ) and interleukin 2 (IL-2) or Th2 cells producing predominantly cytokines interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 10 (IL-10) and interleukin 13 (IL-13). In mice, immunity correlates with a Th1 response. Macrophages infected with *M. tuberculosis*

secrete interleukin 12 (IL-12), which induces the secretion of IFN- γ by CD4 cells and natural killer cells. The IFN- γ enhances the activation of macrophages and improves their ability to prevent the spread of *M. tuberculosis* (Orme, 1993). However, *M. tuberculosis* is not defenceless. It can produce ammonia to counteract phagosomal acidification. Its lypogly can actively scavenge toxic radicals reduced against it by the macrophage (Orme, 1993; Bapela, 2005; Banki *et al*., 1999).

2.1.6. Prevention of tuberculosis

The outcome of mycobacterial infection depends on the host immune response. In most individuals, infection with *M. tuberculosis* induces an immune response sufficient for the protection against progression to the primary disease (Bapela, 2005). Bacille Calmette-Guérin (BCG) vaccine reproduces minimal infection but does not impose a disease risk. BCG vaccine, which is derived from a strain of *M. bovis* attenuated through years of serial passage in culture, was first used in 1921 to protect against TB in humans (Young and Duncan, 1995). Many BCG vaccines are currently administered to 100 million young children each year throughout the world. These vaccines are derived from the original strain but vary in cultural characteristics and ability to induce sensitization to tuberculin. There are differences in techniques and methods of producing them as well as various routes of vaccine administration (Young, 1994).

2.1.7. Infection and symptoms of tuberculosis

TB infection means that the TB germ can be found in the host body, but it cannot always make a person feel sick. This is because of the health immune system which cannot destroy the TB germ by itself, but it can keep the TB germ trapped in the lungs and prevent it from spreading further. Because the TB germ is strong and protects itself with a thick coating, it can remain in an inactive state (dormant) in the body for many years. People with TB infection whose immune systems are weakened and those that have some other kinds of lung disease are more likely to develop TB disease (WHO, 2007). Depending on where in the body the TB bacteria are

multiplying, symptoms of TB disease can vary. Coughing up blood, chest pains, bad cough lasting longer than 2 weeks, weight loss, chills, fever and night sweats are common symptoms of TB disease. Sometimes people experience joint pain like arthritis if the TB is in their bones. Without treatment, a person who has TB disease will infect an average of 10 - 15 people with TB every year (Davies, 2003).

2.1.8. Treatment of tuberculosis

Before effective drugs were available, half of the patients with active pulmonary TB died within 2 years, and only a quarter were cured. With the advent of anti-TB chemotherapy, protracted bed rest and lengthy isolation became unnecessary, and in theory at least, successful treatment was a reasonable goal in all adults (Bartmann, 1988). *Mycobacterium* is naturally resistant to most common antibiotics and chemotherapy agents. This is probably due to their highly hydrophobic cell envelope acting as an efficient permeability barrier. Due to the discovery of the effective antimycobacetrial agents ethambutol (EMB., Figure 2.7), INH, pyrazinamide (PZA., Figure 2.8), RIF and streptomycin (STR., Figure 2.9) between 1950 and 1970s, and reduction in poverty, there was a drastic decrease in the number of TB cases especially in developed countries. However, since 1980s, the number of TB cases throughout the world has been increasing rapidly due to the emergence of MDR-TB (Chan and Iseman, 2002).

The MDR forms of the disease, defined as forms resistant to two or more existing TB-drugs, are often fatal and are difficult and expensive to treat (Basso and Blanchard, 1998; Bastian and Colebunders, 1999). The situation has recently been complicated by the association of TB with HIV in sub-Saharan Africa and many developing countries (Corbett *et al*., 2003; Lurie *et al*., 2004). The situation is exacerbated by the increasing emergence of extensively drug-resistant (XDR) TB (Core Curriculum on Tuberculosis, 2006). Reliable treatment therapy for TB treatment takes a period of 6 - 9 months with the first line TB drugs (EMB, INH, PZA, RIF and STR). In the case of acquired drug resistance only second-line drugs (capreomycin, cycloserine, kanamycin and ethionamide) can be used and these have significant side effects with

approximately 50% cure rate (Gautam *et al*., 2007; Heym and Cole, 1997). The current therapies reduce the pulmonary bacterial burden but the treatment periods of 6 months for non-immune suppressed individuals and at least 9 months for immune suppressed patients are required for reliable treatment efficacy (Bapela, 2005; Quenelle *et al*., 2001). However, floroquinolones such as ofloxacin, norfloxacin can be used which are safer than the above-mentioned second-line drugs but have the disadvantage of being very expensive (Tripathi *et al*., 2005). Emergence of drug-resistant mycobacterial strains is alarming these days. This occurs when a single drug is given alone and when the viable bacterial population in the lesions is large. The occurrence of drug resistance is widely thought to be due to the overgrowth of sensitive organisms by mutant resistant bacilli present in wild strains before they were ever in contact with the drug concerned (Mitchison, 1984). There have been no new anti-TB drugs introduced in the past 30 years. Thus, there is an urgent need to search for and develop new effective and affordable anti-TB drugs (Gautam *et al*., 2007).

2.2. Targets, mode of action of first-line TB drugs

Current chemotherapy for TB largely relies on drugs that inhibit bacterial metabolism with a heavy emphasis on inhibitors of the cell wall synthesis (Zhang, 2005). According to their mode of action, first and second line drugs can be grouped as cell wall inhibitors (INH, EMB, ethionamide, cycloserine), nucleic acid synthesis inhibitors (RIF, quinolones), protein synthesis inhibitors (STR, kanamycin) and inhibitors of membrane energy metabolism (PZA) (Mitchison, 1980). Targets and mechanisms of action of current TB drugs are summarised in Table 2.3.

Existing TB drugs are therefore only able to target actively growing bacteria through the inhibition of cell processes such as cell wall biogenesis and DNA replication. This implies that current chemotherapy is characterised by an efficient bactericidal activity but an extremely weak sterilising activity (defined as the ability to kill the slowly growing or metabolising bacteria that persist after most of the growing bacteria have

Table 2.3. Commonly used first and second line TB drugs and their targets (Zhang, 2005)

Drug (year of discovery)	**MIC (µg/mL)**	**Effect on bacterial cell**	**Mechanisms of action**	**Targets**	**Genes involved in resistance**
Isoniazid (1952)	0.01 - 0.2	Bactericidal	Inhibition of cell wall mycolic acid and other multiple effects on DNA, lipids, carbohydrates and NAD metabolism	Primarily acyl carrier protein reductase (inhA)	*katG*; *inhA*, *ndh*
Rifampicin (1966)	0.05 - 0.5	Bactericidal	Inhibition of RNA synthesis	RNA polymerase β subunit	*rpoB*
Pyrazinamide (1952)	20.0 - 100.0	Bactericidal	Disruption of membrane transport and energy depletion	Membrane energy metabolism	*pncA*
Ethambutol (1961)	1.0 - 5.0	Bactericidal	Inhibition of cell wall arabinogalactan synthesis	Arabinosyl tranferase	*emb*CAB
Streptomycin (1944)	2.0 - 8.0	Bacteriostatic	Inhibition of protein synthesis	Ribosomal S12 protein and 16S rRNA	*rps*L; *rrs* (operon)
Kanamycin (1957)	1.0 - 8.0	Bactericidal	Inhibition of protein synthesis	Ribosomal S12 protein and 16S rRNA	*rps*L*; rrs* (operon)
Quinolones (1963)	0.2 - 4.0	Bactericidal	Inhibition of DNA replication and transcription	DNA gyrase	*gyrA*; *gyr*B
Ethionammide (1956)	0.6 - 2.5	Bacteriostatic	Inhibition of mycolic acid synthesis	Acyl carrier protein reductase (inhA)	*inh*A; *eta*A/*ethA*
Para-aminosalicylic acid (1946)	1.0 - 8.0	Bacteriostatic	Inhibition of folic acid and iron metabolism	Unknown	Unknown
Cycloserine (1952)	5.0 - 20.0	Bacteriostatic	Inhibition of peptidoglycan synthesis	D-alanine racemase	*alr*A; *Ddl*

been killed by bactericidal drug). Sterilising activity also describes the ability to eliminate latent or dormant bacteria that survive inside the host macrophages (Mitchison, 1980). Although achieving a clinical cure, the current TB chemotherapy does not achieve a bacteriological-eradication of all bacilli in the lesions (McCune and Tompsett, 1956).

2.2.1. Isoniazid (INH)

INH (Figure 2.5) is the synthetic hydrazide of isonicotic acid discovered in 1952 and the first-line antituberculosis medication used in the prevention and treatment of TB. INH is the cornerstone of the therapy and should be included in all regimens unless a high degree of INH resistance exists. This drug is never used on its own to treat TB because resistance develops quickly. INH is highly selective and acts almost exclusively against *M. tuberculosis*, *M. bovis* and *M. africanum*. This remarkable selectivity in its action is thought to be mediated by the bacterial enzyme catalase peroxidase which catalyses the reaction converting INH to a potent bactericidal derivative. INH is bactericidal at MIC levels of less than 0.1 µg/mL for 80% of susceptible strains of *M. tuberculosis* (Reichman and Hershfield, 2000). INH is available in tablet, syrup and injectable forms (given intramuscularly and intravenously).

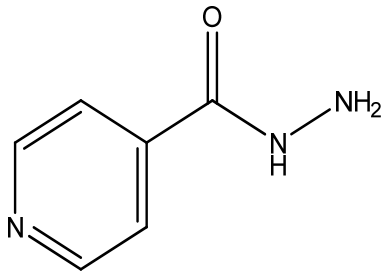


Figure 2.5. Chemical structure of isoniazid (INH)

2.2.1.1. Mechanism of action

The exact mechanism of the action of INH has not been fully elucidated, but several

mechanisms including interference with the metabolism of bacterial proteins, nucleic acids, carbohydrates and lipids have been proposed. INH is a prodrug and must be activated by bacterial catalase. It is activated by catalase-peroxidase enzyme katG to form isonicotinic acyl anion or radical. These forms will then react with a NADH radical to form isonicotinic acyl-NADH complex. This complex will then binds tightly to ketoenoylreductase known as InhA and prevent access of the natural enoyl-AcpM substrate. This mechanism inhibits the synthesis of mycolic acid in the mycobacterial cell wall. INH combines with an enzyme which interferes with the cell metabolism of the bacteria. As a result of the disruption in its metabolism and without a cell wall the bacteria die (Bapela, 2005). INH reaches therapeutic concentration in serum, cerebrospinal fluid (CSF) and within caseous granulomas and metabolised in the liver via acetylation. INH is bactericidal to rapidly-dividing mycobacteria, but is bacteriostatic if the *Mycobacterium* is slow-growing. Susceptible bacteria may undergo 1 or 2 divisions before multiplication is arrested (Bartmann, 1988).

2.2.1.2. Resistance to INH

INH inhibits the biosynthesis of mycolic acids present in the cell wall of *M. tuberculosis*. This renders the mycobacterial cell wall defective, thereby penetratable to toxic oxygen. KatG is the only enzyme in *M. tuberculosis* capable of activating INH. Expression of either the KatG or an alkyl hydroperoxidase AhpC is considered sufficient to protect the bacilli against toxic peroxides (Wilson and Collins, 1996).

2.2.1.3. Side effects and toxicity

Adverse reactions include rash, abnormal liver functions, hepatitis, sideroblastic anemia, peripheral neuropathy, mild central nervous system (CNS) effects and drug interactions resulting in increased phenytoin (dilantin) or disulfiran (antabuse) levels. Peripheral neuropathy and CNS effects are associated with the use of INH and are due to pyridoxine (vitamin B6) depletion. Headache, poor concentration, poor memory and depression have all been associated with INH use. The frequency of these side effects is not known and the association with INH is not well validated. The presence

of these symptoms is not frequently disabling and is not a reason to stop treatment with INH and the patients are strongly encouraged to continue treatment despite these symptoms (Holdiness, 1984).

The hepatoxicity associated with INH results from the toxic effect of an intermediate product produced by N-hydroxylation of monoacetylhydrazine, one of the metabolites of INH, by the liver cytochrome P-450 mixed function oxidase system. Hepatoxicity, nausea, vomiting, abdominal pains and appetite loss can be avoided with close clinical monitoring of the patient (Holdiness, 1984).

2.2.2. Rifampicin (RIF)

RIF (Figure 2.6) is the second major antituberculosis agent which is used in conjunction with other antituberulosis agents in the treatment of TB. RIF is a semi synthetic derivative of one of a group of structurally similar, complex macrocyclic antibiotics produced by *Streptomyces mediterranei* (Bartmann, 1988).

RIF inhibits the growth of most Gram-positive bacteria as well as many Gram-negative bacteria. RIF inhibits *M. tuberculosis* at concentrations ranging from 0.005 – 0.2 µg/mL *in vitro* (Bapela, 2005; Duman *et al*., 2004). RIF is soluble in organic solvents and in water at acidic pH (Bapela, 2005).

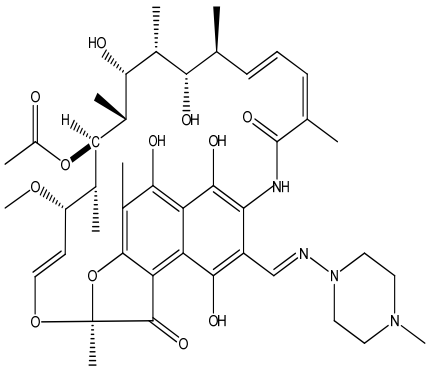


Figure 2.6. Chemical structure of rifampicin (RIF)

2.2.2.1. Mechanism of action

RIF may be bacteriostatic or bactericidal in action, depending on the concentration of the drug attained at the site of infection and the susceptibility of the infecting organism. RIF inhibits dioxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA)-polymerase of the *Mycobacterium* by forming a stable drug-enzyme complex, leading to the suppression of the initiation of chain formation in RNA synthesis (Bapela, 2005). More specifically, the β–subunit of this complex enzyme is the site of the action of the drug, although RIF binds only to the holoenzyme (Bartmann, 1988).

2.2.2.2. Resistance to RIF

Mutation in RNA polymerase beta subunit gene (rpoB) is the major mechanism of resistance to RIF with high frequencies of 90% or more (Taniguchi, 2000). Evaluation of the relationship between RIF's susceptibility and genetic alteration in rpoB gene also showed that 95% of the RIF-resistant *M. tuberculosis* isolates involved genetic alterations in an 81-base pair core region of rpoB gene. This region is called the rifampicin resistance-determining region (Ramaswammy and Musser, 1998). Moreover, these genetic alterations in the rpoB gene are suspected as being the resistance mechanisms to RIF (Taniguchi, 2000).

2.2.2.3. Side effects and toxicity

In humans, acute overdose with RIF, i.e. up to 12g has not been fatal, however one fatality has been reported following ingestion of a single 60g dose of the drug (AHFS Drug Information, 2000). The lethal dose (LD_{50}) of RIF in mice is 0.885 g/kg. The most important complication of RIF is liver toxicity, which occurs 4 times more frequently in regimens containing both INH and RIF than in those containing INH alone (Bartmann, 1988., Bapela, 2005).

2.2.3. Ethambutol (EMB)

EMB (Figure 2.7) is a synthetic antituberculosis agent prescribed to treat TB. It is active *in vitro* and *in vivo* against *M. tuberculosis*, *M. bovis*, *M. marinum*, *M. avium* and *M. intracellulare*. EMB is usually given in combination with other drugs such as INH, RIF and PZA, at a daily dose of 25 mg/kg to humans during the first 2 months of well-supervised therapy and at 15 mg/kg for longer, often less well supervised periods (Bartmann, 1988).

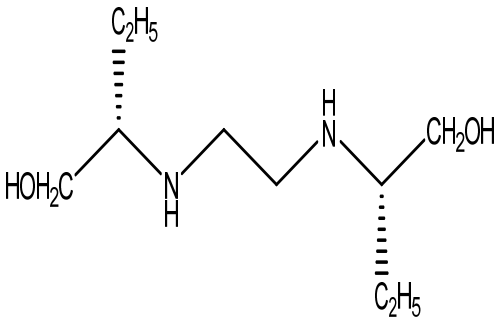


Figure 2.7. Chemical structure of ethambutol (EMB)

2.2.3.1. Mechanism of action

EMB may be bacteriostatic or bactericidal in action, depending on the concentration of the drug attained at the site of infection and the susceptibility of the organism. Although the exact mechanism has not yet been fully determined, the drug appears to inhibit the synthesis of one or more metabolites in susceptible bacteria resulting in the impairment of cellular metabolism, arrest of multiplication, and cell death. EMB is active against susceptible bacteria only when they are undergoing cell division (AHFS Drug Information, 2000).

2.2.3.2. Resistance to EMB

EMB has been shown to inhibit the incorporation of mycolic acids into the cell wall. It has also been shown to inhibit the transfer of arabinogalactan into the cell wall of

mycobacteria (Ramaswamy and Musser, 1998). Only the dextroisomer of EMB is biologically active, an observation consistent with the idea that the drug binds to a specific drug target, which is assumed to be arabinosyl transferase (Ramaswammy and Musser, 1998). The EmbB gene, encoding arabinosyl transferase which catalyses cell wall synthesis, is mutated in EMB-resistant strains (Taniguchi, 2000).

2.2.3.3. Side effects and toxicity

A single administration of EMB has low toxicity in mice (Diermeier *et al*., 1966). In humans the adverse effects of EMB include dermatitis, pruritis, headache, dizziness, fever and mental confusion.

2.2.4. Pyrazinamide (PZA)

PZA (Figure 2.8) is a derivative of niacinamide and is a synthetic antituberculosis drug used to treat TB in patients (AHFS Drug Information, 2000). Currently, PZA is considered as a first line drug, and only used in combination with other drugs such as INH and RIF in the treatment of *M. tuberculosis*. PZA has no other medical uses and is not used to treat other mycobacteria (*M. bovis* and *M. leprae*) which are resistant to the drug. PZA is used in the first two to four months of treatment to reduce the duration of treatment required.

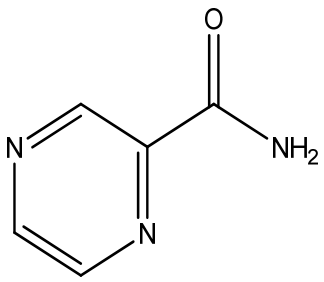


Figure 2.8. Chemical structure of pyrazinamide (PZA)

2.2.4.1. Mechanism of action

PZA may be bacteriostatic or bactericidal in action, depending on the concentration of the drug attained at the site of infection and the susceptibility of the organism (Bapela, 2005). The drug is active *in vitro* and *in vivo* at a slightly acidic pH. PZA stops the growth of *M. tuberculosis*, which has an enzyme pyrazinamidase that is only active at acidic pH. PZA converts the enzyme to the active form, pyrazinoic acid (POA). POA inhibits the enzyme fatty acid synthetase 1, which is required by the bacterium to synthesise fatty acids.

In addition, POA lowers the pH of the environment below a certain level which is optimal for *M. tuberculosis* growth. Mutations of the pyrazinamidase gene (*pncA*), are responsible for PZA resistance in *M. tuberculosis*. This appears to contribute to the drug's antimycobacterial activity *in vitro* (Bartmann,1988).

2.2.4.2. Resistance to PZA

The mechanism of action and the resistance of *M. tuberculosis* to PZA have also been partially identified. PZA is crucial for achieving sterilization by killing persisting semi-dormant bacilli in the lungs. Its activity depends on the presence of a bacterial amidase, which converts PZA to POA, which is the active antibacterial molecule (Mitchison and Selkon, 1996). PZA-resistant bacilli lack this amidase activity. The pncA gene encoding for this has been identified and the mutation to this pncA gene has been associated with resistance to PZA (Scopio and Zhang, 1996).

2.2.4.3. Side effects and toxicity

The most frequent adverse effect of PZA is hepatoxicity. Hepatoxicity becomes a problem when PZA is given in large doses and for long periods. Hepatoxicity may appear at any time during therapy. When PZA is used in short-course therapy no increase in the incidence of hepatotoxicity is noted (Bapela, 2005). The original dose for PZA was 40 – 70 mg/kg and the incidence of drug-induced hepatoxicity has fallen

significantly since the recommended dose has been reduced. In the standard four-drug regime (INH, RIF, PZA and EMB), PZA is the most common cause of drug-induced hepatitis. It is not possible to clinically distinguish pyrazinamide-induced hepatitis from hepatitis caused by INH or RIF.

Another common side effect of PZA is joint pains (arthralgia), which can be distressing to patients, but never harmful. Other side effects include nausea and vomiting, anorexia, sideroblastic anemia, skin rash, urticaria, pruritus, hyperuricemia, dysuria, interstitial nephritis, malaise, rarely porphyria and fever (British Thoracic Society, 1984). In mice, PZA has a LD_{50} of 3.4 g/kg when administered orally (Robinson *et al*., 1954).

2.2.5. Streptomycin (STR)

STR (Figure 2.9) is an aminoglycoside antibiotic which is particularly active against *M. tuberculosis* as well as against many Gram-negative bacteria. STR is bactericidal for the tubercle bacillus *in vitro*. Concentrations as low as 0.4 µg/mL inhibit growth of the tubercle bacillus *in vitro*. STR is an alternative to EMB in the four-drug protocols for the treatment of TB. STR is easily soluble in water (Bapela, 2005).

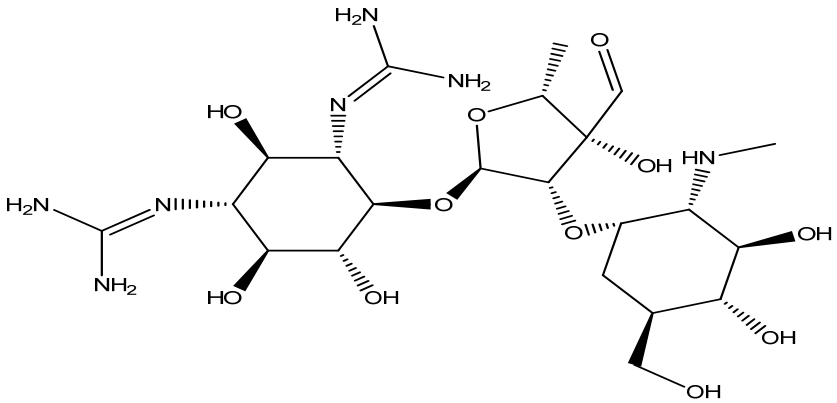


Figure 2.9. Chemical structure of streptomycin (STR)

2.2.5.1. Mechanism of action

STR, like other aminoglycosides, is actively transported across the bacterial cell membrane by an oxygen-dependent system. Once inside the bacteria, it binds to the polysomes and inhibits the synthesis of proteins. The drug binds to the 30S subunit of the bacterial ribosome which consists of 21 proteins and a single 16S molecule of RNA. Protein synthesis in the bacteria is blocked by inhibiting the movement of the peptidyl-Trna associated with translocation and this stimulates tRNA errors (Bapela, 2005).

2.2.5.2. Resistance to STR

Most STR resistance strains have a mutation on the rrs and rspL genes encoding a 16S rRNA and a 12S ribosomal subunit protein respectively (Taniguchi, 2000). In contrast to other bacteria, which have multiple copies of rRNA genes, *M. tuberculosis* complex members have only one copy. Hence, single nucleotide changes can potentially produce antibiotic resistance (Ramaswammy and Musser, 1998).

2.2.5.3. Side effects and toxicity

The toxic effects of STR are manifested mainly on vestibular rather than auditory function in human beings. An acute toxic effect following intracisternal injection in animals is clonic-convulsion. Other acute toxic effects following cutaneous or intravenous injections are nausea, vomiting and ataxia (Holdiness, 1984).

2.3. Why are new TB drugs needed?

HIV/AIDS has dramatically increased the risk of developing active TB and HIV co-infection makes TB more difficult to diagnose and treat. The increasing emergence of MDR-TB and the nature of persistent infections pose additional challenges to the treatment with conventional anti-TB drugs. Although TB can be treated with current

drugs, treatment is complex and long involving 4 drugs for 2 months and 2 more drugs for at least another 4 months. Direct Observed Treatment (DOT) as promoted by the WHO to improve compliance for the difficult and long regimen can improve cure rates, but is demanding for patients and labour intensive for health staff (O'Brien and Nunn, 2001).

In pioneering studies McDermott and colleagues showed that the efficacy of drugs against *M. tuberculosis in vitro* was not matched by their efficiency *in vivo* (McCune and Tompsett, 1956). The exponentially growing cultures of *M. tuberculosis* can be sterilised using frontline bactericidal drugs such as INH and RIF, yet the same drug combination requires months to achieve similar effects against bacteria living in host tissues. This is because of the failure of the drugs to achieve optimal levels within TB lesions, but there is evidence that drug availability is not a limiting factor (Barclay *et al.*, 1953; Clark, 1985). It has been proposed that persistence of tubercle bacilli, which consists of 4 different populations, in the chemotherapy treatment might be attributable to physiologic heterogeneity of the bacteria in the tissues (Mitchison, 1979).

These populations are:

- Bacteria that are actively growing are killed primarily by INH.
- Bacteria that have spurts of metabolism are killed by RIF.
- Bacteria that are characterised by low metabolic activity and reside in acid pH environment are killed by PZA.
- Bacteria that are dormant or persisters are not killed by any current TB drug.

This idea was supported by the long established observation that slow and non-growing bacteria are phenotypically resistant or tolerant to killing by antimicrobials (Handwerger and Tomasz, 1985).

During the initial phase of chemotherapy treatment, which lasts about 2 days, the bacilli are killed exponentially at a rapid rate, followed by a further lengthy period of

much slower exponential killing. It is assumed that those bacilli that are killed in the first 2 days are actively multiplying, while those in the succeeding period are persisters killed by the slower sterilising activities of the drugs (Jindani *et al*., 2003). In an *in vitro* model of drug action, a 30-day static culture has been extensively used for the last 60 years and has been taken to resemble the persister population in its response to the drugs (Mitchison and Selkon, 1956; Mitchison, 1992; Herbert *et al*., 1996). The drugs added to this static culture have the same slow sterilising actions that are responsible for the prolongation of therapy. This evidence suggests that activity against the population of persistent bacilli ultimately determines the duration of therapy necessary to provide a stable cure of the host (Grosset and Ji, 1998).

Evidently there is an urgent need to develop new and more effective TB drugs that are not only active against MDR-TB but also shorten the length of treatment and target the non-replicating persistent bacilli.

2.4. New TB drugs in the pipeline

Drug development for TB and other diseases has been at a standstill for decades. Today thanks to, the Global Alliance for TB Drug Development (TB Alliance), which was created in 2000 and funded by the Bill and Melinda Gates Foundation, the TB drug pipeline is richer than it has been in the last 40 years. This TB alliance focuses on both pre-clinical and clinical development of candidate compounds for TB chemotherapy and is associated with projects aimed to identify compounds currently being developed. In addition to this, increased public awareness on the lack of research and development for neglected diseases have led in recent years to some multinational pharmaceutical companies up setting Research and Development (R&D) institutes on a ‘no-profit-no-loss’ basis for drug development for TB, malaria and leishmaniasis. Among the multinational pharmaceutical companies currently involved in anti-TB drug R&D are: Novartis, AstraZeneca and GlaxoSmithKline (GSK). Smaller pharmaceutical companies have also engaged in neglected disease R&D on a commercial basis and with some success as two of the anti-TB candidate

drugs currently in clinical trials have been developed by medium-size pharmaceutical companies such as Lupin Limited (India) and Otsuka Pharmaceuticals (Japan., Moran *et al*., 2005).

The Global TB drug pipeline as reported by the Stop TB partnership working group on new TB drugs is summarised in Figure 2.10 and Table 2.4. This is an overview of all drug candidates in the pipeline, belonging to different entities and not only the TB Alliance. In order to analyse the pipeline, the drug candidates are grouped in two main categories: novel chemical entities and compounds originating from existing families of drugs where novel chemistry is used to optimise the compounds.

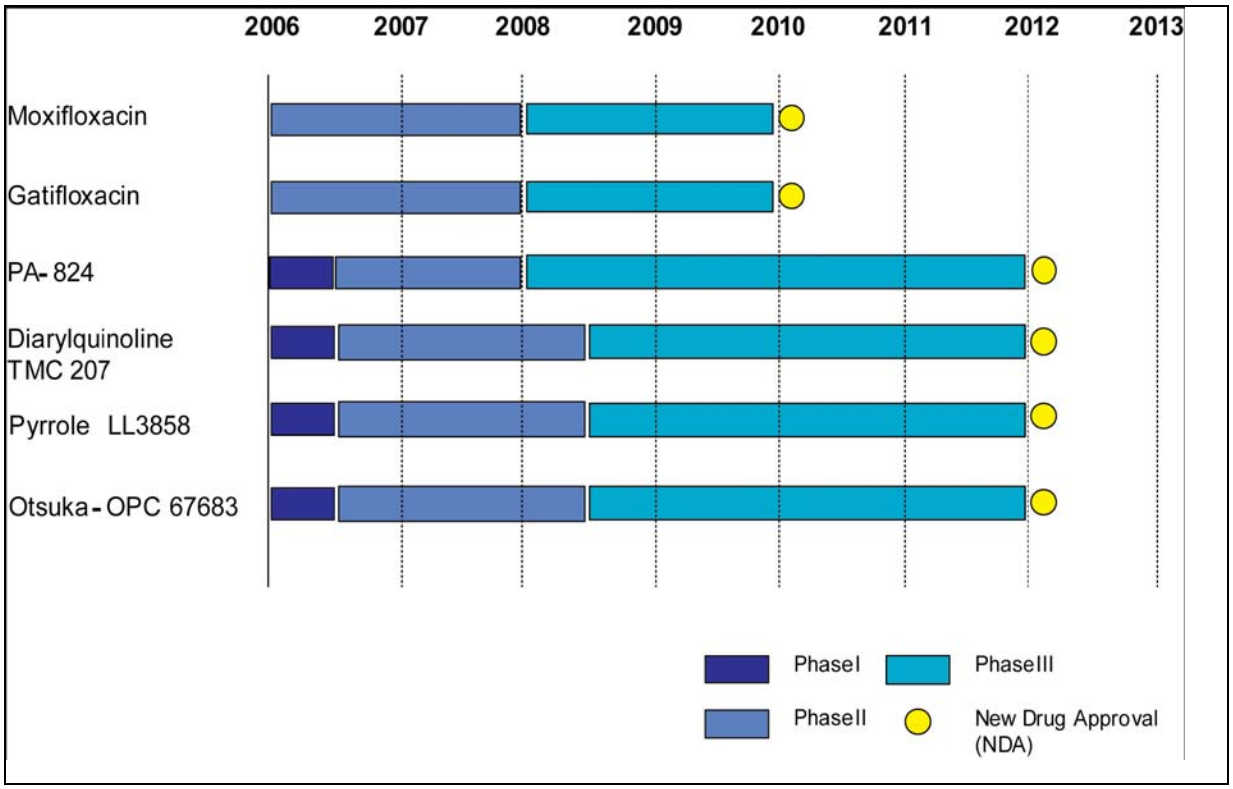


Figure 2.10. Expected time lines towards approval of candidate drugs currently in clinical stages of development (Global TB Alliance Annual report 2004 - 2005)

Table 2.4. Global TB drug pipeline, March 2006 (provided by Stop TB Partnership working on new TB drugs)

Discovery		Preclinical	Clinical
Thiolectomycin Analog NIAID, NIH	**Nitrofuranylamides** NIAID, University of Tennessee	**Diamine SQ-109** Sequella Inc	**Diarylquinoline TMC207** Johnson & Johnson
Cell Wall Inhibitors Colorado State University, NIAID	**Nitroamidazole Analogs** NIAID, Novartis Institute for Tropical Diseases TR Alliance (University of Auckland)	**Dipiperidines SQ-609** Sequella Inc.	**Gatifloxacin** OFLOTUB Consortium, Lupin, NIAID TBRU, Tuberculosis Research Centre, WHO-TDR
Dihydrolipoamide Acyltransferase Inhibitors Cornell University, IAID	**Focused Screening** GlaxoSmithKline, TB Alliance	**Nitroimidazo-oxazole** Otsuka	**Moxifloxacin** Bayer Pharmaceuticals, CDC TBTC, Johns Hopkins University, NIAID TBRU, TB Alliance
InhA Inhibitors GlaxoSmithKline, TB Alliance	**Picolinamide Imidazoles** NIAID, TAACF	**Synthase Inhibitor FAS20013** FasGEN Inc.	**Nitroimidazole PA-824** Chiron Corporation, TB Alliance
Isocitrate Lyase Inhibitors (ICL) GlaxoSmithKline, TB Alliance	**Pleuromutilins** GlaxoSmithKline, TB Alliance	**Translocaase Inhibitors** Sequella Inc. Sankyo	**Nitroimidazo-axazole OPC-67883** Otsuka
Macrolides TB Alliance, University of Illinois	**Quinolones** KRICT/Yonsel University, NIAID, TAACF, TB Alliance	**Non-Fluorinated Quinolones** TaiGen	**Pyrrole LL-3858** Lupin Limited
Methyltransferase Inhibitors Anacor Pharmaceuticals	**Screening and Target identification** AstraZeneeca		
Natural Products Exploration BIOTEC, California State University, ITR, NIAID			

2.4.1. Novel chemical entities

2.4.1.1. Diarylquinoline TMC_{207}

The diarylquinoline TMC_{207} (Table 2.4, Figure 2.11), an extremely promising member of a new class of anti-mycobacterial agents, has a potent early and late bactericidal activity in the non-established infection in murine TB model exceeding that of INH. The substitution of RIF, INH or PZA with diarylquinoline TMC_{207} accelerated activity leading to complete culture conversion after 2 months of treatment in some combinations.

The diarylquinoline-isoniazid-pyrazinamide with diarylquinoline-rifampicin-pyrazinamide combinations cleared the lungs of TB in all mice after 2 months. Diarylquinoline TMC_{207} also has been tested in various combinations with the second line drugs such as amikacin, PZA, moxifloxacin and ethionamide in mice infected with the drug-susceptible virulent *M. tuberculosis* H_{37}RV strain (Adries *et al*., 2005). The target and mechanism of action of diarylquinoline TMC_{207} is different from those of other anti-TB agents implying low probability of cross-resistance with existing TB drugs (Adries *et al*., 2005).

It is further suggested that diarylquinoline TMC_{207} is able to inhibit bacterial growth, when tested on MDR-TB isolates, by inhibiting ATP synthase leading to ATP depletion and pH imbalance (Adries *et al*., 2005., Petrella *et al*., 2006). About 20 molecules of this agent have been shown to have an MIC of below 0.5 µg/mL against *M. tuberculosis* H_{37}RV strain. Antimicrobial activity was confirmed *in vivo* for three of these molecules. A thorough assessment of diarylquinoline activity against MDR-TB *in vivo* would however require testing of animal models infected with multi-drug resistant bacterial strains rather than with drug-susceptible strains (Lounis *et al*., 2006). Diarylquinoline TMC_{207} is currently in Phase II Clinical Trials (Figure 2.10).

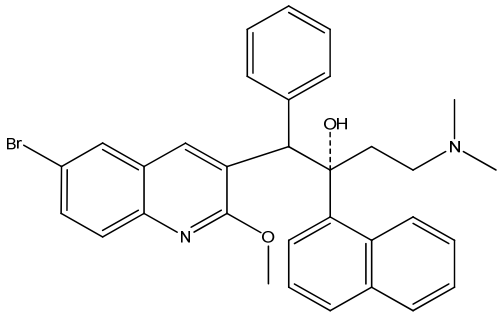


Figure 2.11. Chemical structure of diarylquinoline TMC_{207}

2.4.1.2. Nitroimidazole PA-8_{24}

Nitroimidazole PA-8_{24} (Figure 2.12), is a new nitroimidazole derivative developed by PathoGenesis-Chiron in 1995 and currently being developed by the TB Alliance. The TB Alliance received worldwide exclusive rights to PA-8_{24} and its analogs for the treatment of TB. PA-8_{24} entered Phase I clinical trials in June 2005 (Casenghi, 2006).

In vitro, PA-8_{24} showed high activity against drug-sensitive and drug-resistant *M. tuberculosis* strains, indicating that there is no cross-resistance with current TB drugs (Stover *et al*., 2000). Experiments performed on mice showed that the administration of PA-8_{24} at doses ranging from 25.0 - 100.0 mg/mL produced reductions in the bacterial burden in the spleen and lungs when compared to that produced by INH at 25 mg/mL (Stover *et al*., 2000., Tyagi *et al*., 2005).

Further investigations are required to assess the potentiality of PA-8_{24} to improve the treatment of both drug-susceptible and multi-drug resistant tuberculosis when used in novel combinations with new drug candidates in addition to existing antituberculosis drugs. Nitroimidazole PA-8_{24} is currently in Phase II Clinical Trials (Figure 2.10).

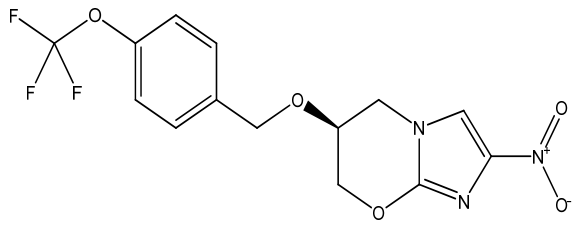


Figure 2.12 Chemical structure of Nitroimidazole PA-8_{24}

2.4.1.3. Nitroimidazole OPC-$6_7 68_3$

Nitroimidazole OPC-$6_7 68_3$ (Figure 2.13) belongs to a subclass of mycolic acid inhibitors, which interferes with the biosynthesis of the mycobacterial cell wall. MIC's of this compound were determined using standard and clinical isolated *M. tuberculosis* strains, including MDR strains. *In vitro*, OPC-$6_7 68_3$ showed high activity against drug-sensitive as well as drug-resistant strains with MIC's ranging from 6.0 - 24.0 mg/mL and also strong intracellular activity against *M. tuberculosis* H_{37}RV strain residing within human macrophages (Casenghi, 2006). Studies in animal models showed that OPC-$6_7 68_3$ is effective against sensitive H_{37}RV and MDR-TB strains *in vivo* starting from a concentration of 0.03125 mg/body (Casenghi, 2006).

The TB Alliance is currently negotiating with Otsuka Pharmaceuticals concerning the further joint development of this compound. OPC-$6_7 68_3$ is in Phase II Clinical Trials (Casenghi, 2006., Table 2.4; Figure 2.10).

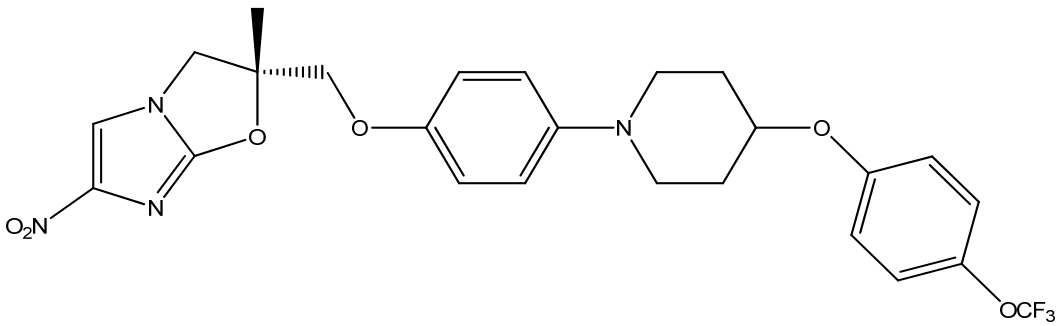


Figure 2.13. Chemical structure of Nitroimidazole OPC-$6_7 68_3$

2.4.1.4. Pyrrole LL- $_{3}8_{5}8$

Very little information on the development of pyrroles as anti-mycobacterial agents is currently available (Casenghi, 2006). Pyrroles derivatives were found to be active against standard and drug-sensitive *M. tuberculosis* strains *in vitro* (Deidda *et al*., 1998., Ragno *et al*., 2000). Lupim Limited reported the identification of a pyrrole derivative (LL-$_{3}8_{5}8$) that showed higher bactericidal activity than INH when administered as monotherapy to infected mice. In mice models, a 12 weeks treatment with LL-$_{3}8_{5}8$ plus INH and RIF, or LL-$_{3}8_{5}8$ plus INH-RIF-PZA, sterilized the lungs of all infected mice. Experiments conducted in mice and dogs showed that the compound is well absorbed, with levels in serum above the MIC. No information is available concerning the molecular mechanisms that mediate LL-$_{3}8_{5}8$'s bacterial activity (Casenghi, 2006). Pyrrole LL- $_{3}8_{5}8$ is in Phase II Clinical Trial (Figure 2.10).

2.4.1.5. Pleuromutilins

The pleuromutilins represent a novel class of antibiotics derived from a natural product. They interfere with protein synthesis by binding to the $_{23}$S rRNA and therefore inhibiting the peptide bond formation (Schlunzen *et al*., 2004). Recent studies showed that cross-resistance might occur among pleuromutilins and oxazolidinones (Long *et al*., 2006). Pleuromutilins have also showed to inhibit the growth of *M. tuberculosis in vitro* (Casenghi, 2006)

2.4.1.6. Dipiperidine SQ-6_{09}

Dipiperidine SQ-6_{09} is a novel compound structurally unrelated to existing anti-TB drugs. It kills *M. tuberculosis* by interfering with cell wall biosynthesise. Antimicrobial activity has been demonstrated *in vivo* in mice models (Nikonenko *et al*., 2004).

2.4.1.7. ATP Synthase Inhibitor FAS_{20013}

FAS_{20013} is a novel compound identified by Fasgen. It belongs to the class of β-sulphonylcarboxamides. Fasgen claims that FAS_{20013} will kill more organisms in a 4 hour exposure that INH or RIF can during a 12 -14 day exposure (Casenghi, 2006). The compound is very effective in killing MDR-TB organisms that are resistant to the multiple drugs currently in use. A series of recent laboratory experiments indicate the superior effect of FAS_{20013} as compared to current drugs in terms of its ability to sterilize TB lesions and kill latent TB. Therapeutic evaluation of FAS_{20013} has repeatedly shown its effectiveness in mice, but it appears to or has no serious side effects. The compound is up to 100% bio-available when administered orally. To date no dose-limiting toxicity has been encountered, even when doses are 10 times administered. The compound is thought to act through inhibition of ATP synthase, however the available publications assessing the efficacy of this compound are of poor quality (Casenghi, 2006., Jones *et al*., 2000., Parrish *et al*., 2004).

2.4.1.8. Translocase I inhibitor

These are compounds which specifically inhibit mycobacterial translocase I, an enzyme required for bacterial cell wall. Preclinical evaluations of the compounds are planned (Casenghi, 2006).

2.4.1.9. InhA Inhibitors

Frontline drugs such as INH target the enoyl reductase enzyme InhA, found in *M. tuberculosis* which catalyses the last step in the fatty acid synthase pathway (FAS II). Drug resistance to INH results primarily from KatG (the enzyme that activates INH), therefore the InhA inhibitors that do not require activation by KatG are attractive candidates in the search for new drugs. The main purpose is to bypass the activation step and directly inhibit InhA. A possible limitation for this kind of compound is that cross-resistance with INH may easily occur (Casenghi, 2006., Banerjee *et al*., 1994).

2.4.1.10. Isocitrate Lyse Inhibitors

The isocitrate lyase (ICL) enzyme has been shown to be essential for long-term persistence of *M. tuberculosis* in mice, but not required for bacilli viability in normal culture (McKinney *et al*., 2000). McKinney and collaborators have shown that inhibition of ICL1 and ICL2 (the two isoforms of isocytrate lyase present in *M. tuberculosis*), blocks the growth and survival of *M. tuberculosis* in macrophages and in mice at an early and late stage of infection (McKinney *et al*., 2000). GSK planned in 2000 to screen 400 000 ICL inhibitors as potential therapeutic drugs. Up to now 900 000 compounds have been screened but no successful inhibitors have been identified. (Casenghi, 2006). The structure of the ICL active site makes the screening of inhibitors lengthy and the active site of this enzyme appears not to be easily reached by compounds (Casenghi, 2006).

2.4.2. Chemicals originating from existing families of drugs

2.4.2.1. Fluoroquinolones

Fluoroquinolones were introduced into clinical practice in the 1980's. They are characterised by broad spectrum antimicrobial activity and are recommended and widely used for the treatment of bacterial infection of the respiratory, gastrointestinal and urinary tracts (Bartlett *et al*., 2000., Neu, 1987). Fluoroquinolones have been also found to have activity against *M. tuberculosis* and are currently part of the recommended regimen as second line drugs (Grosset, 1992., Tsukamura *et al*., 1985). Since fluoroquinolones share the same molecular targets, it is highly probable that they will trigger the same mechanisms of resistance. The major concern is that widespread use of fluoroquinolones for treatment of other bacteria infections may select for resistant strains of *M. tuberculosis*.

In a study conducted in USA and Canada, among referral samples isolates between 1996 and 2000, resistance to ciprofloxacin was assessed and was found to occur in 1.8% of isolates and 75.8% were also MDR. The authors concluded that despite the

widespread use of fluoroquinolones for treatment of common bacterial infection in USA and Canada, resistance to fluoroquinolones remains rare and occurs mainly in MDR strains (Bozeman *et al*., 2005). In contrast, in a different study conducted by Ginsburg and collaborators between 1998 and 2002, the incidence of *M. tuberculosis* fluoroquinolone resistance in a small sample of patients with newly diagnosed TB was found to be high among patients with prior fluoroquinolone exposure (Ginsburg *et al*., 2003b). The risk of selecting fluoroquinolones-resistant *M. tuberculosis* strains by empirically treating with fluoroquinolones for presumed infections before a diagnosis of TB established is of great concern. For this reason, some investigators in the TB field argue that the use of fluoroquinolones might be better reserved for specific serious infection such as TB rather than becoming the workhorse of antimicrobial treatment, however given the current widespread use of quinolones this might not be realistic (Bozeman *et al*., 2005).

The interest in fluoroquinolones as antituberculosis agents has focused on the new fluoroquinolones moxifloxacin and gatifloxacin. Despite a lack of a comprehensive work comparing the activities of old and new classes of fluoroquinolones in *M. tuberculosis*, what can be inferred from published sources are that moxifloxacin and gatifloxacin are characterised by a higher activity against *M. tuberculosis in vitro* when compared to the old fluoroquinolones ofloxacin and ciprofloxacin (Hu *et al*., 2003; Paramasivan *et al*., 2005; Rodriguez *et al*., 2001; Sulochana *et al*., 2005).

2.4.2.1.1. Gatifloxacin

Gatifloxacin (GAT, Figure 2.14) is a new fluoroquinolone marketed in the U.S. by Bristol-Myers Squibb as Tequin. GAT has been found to have *in vitro* and *in vivo* bactericidal activity against *M. tuberculsosis* (Hu *et al*., 2003). In an *in vivo* study, GAT showed the highest bactericidal activity during the first 2 days but not thereafter (Paramasivan *et al*., 2005). Similar results were obtained when GAT was used in combination with INH or RIF, GAT was able to slightly increase the bactericidal activity of INH or RIF only during the first 2 days (Paramasivan *et al*., 2005). One paper reported that when tested in mice in combination with ethionamide and PZA

(high doses: 450 mg/kg, 5 days per week) GAT was able to clear lungs of infected mice after 2 months of treatment (Cynamon and Sklaney, 2003).

Current available data on GAT does not support the hypothesis that introduction of GAT with first-line drugs will impressively contribute to shorten TB treatment. Further investigation should be done to properly assess the activity of GAT *in vitro* and in animal models. GAT is currently in Phase II Clinical Trials (Figure 2.10), conducted under the supervision of the European Commission Oflotub Consortium (Hu *et al*., 2003). The aim of the trial is to evaluate the efficacy and safety of a four months gatifloxacin-containing regimen for treatment of pulmonary TB (Hu *et al*., 2003).

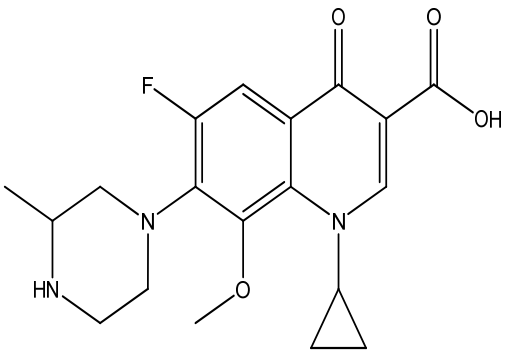


Figure 2.14. Chemical structure of gatifloxacin (GAT)

2.4.2.1.2. Moxifloxacin

Moxifloxacin (MXF, Figure 2.15) is produced by Bayer Pharmaceuticals and marketed as Avelox in the USA. MXF is the most promising of the new fluroquinolones being tested against *M. tuberculosis* (Miyazaki *et al*., 1999). *In vitro*, MXF appeared to kill a subpopulation of tubercle bacilli not killed by RIF, i.e. rifampicin-tolerant persisters, while other fluoroquinoles ciprofloxacin and ofloxacin did not have any significant bactericidal effect on the same subpopulation (Hu *et al*., 2003). One possibility is that MXF interferes with protein synthesis in slowly metabolising bacteria, through a mechanism that differs from that used by RIF.

In mice models, the activity of MXF against tubercle bacilli was comparable to that of INH (Miyazaki *et al*., 1999).When used in combination with MXF and PZA, MXF has been reported to kill the bacilli more effectively that the INH+RIF+PZA combination. In conclusion, *in vitro* and *in vivo* studies suggest that MXF might be a promising candidate drug to shorten TB treatment (Grosset *et al*., 1992). MXF is currently in Phase III Clinical Trials (Figure 2.10). A trial substituting EMB with MXF during intensive phase was initiated before the animal models and showed no advantage over EMB (Miyazaki *et al*., 1999).

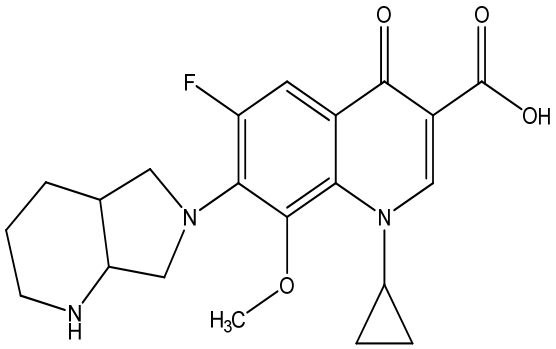


Figure 2.15. Chemical structure of moxifloxacin (MXF)

2.4.2.2. New quinolones

In 2003, the TB Alliance launched a project in collaboration with the Korean Research Institute of Chemical Technology (KRCT) and the Yonsei University aimed to synthesise and evaluate novel and more effective quinolone compounds that could shorten first-line treatment. To date, 450 compounds have been synthesised and tested for their antituberculosis activity (Flamm *et al*., 1995). During the research, the sub-class termed 2-pyridones was identified as being the one showing the most potent activity against *M. tuberculosis* in both its growing and persistent states. This sub-class of compounds was already identified by Abbott in 1998 and found to have activity against drug-susceptible and drug-resistant *M. tuberculosis*. The lead compounds identified so far, showed better activity than GZF and MXF (Oleksijew *et al*., 1998).

2.5. Discussion and Conclusion

According to the WHO Global TB Report of 2006, South Africa has an estimated number of nearly 340,000 new TB cases per year with an incidence rate of 718 cases per 100,000. The TB epidemic in South Africa is exacerbated due to HIV/AIDS with an estimated 60% of adult TB patients being HIV positive. MDR-TB is further exacerbating the epidemic (WHO, 2007 Fact Sheet 104). Early stage drug discovery is a key in the pipeline to find novel drugs for TB. While the existence of a TB drug pipeline, after decades of virtually no new TB drug, is welcome, there are still far too few compounds that represent new chemical classes with novel mechanisms of action and a low probability of encountering pre-existing drug resistance. Of the approximately 40 compounds in the current pipeline, it is unlikely that a useful therapy will emerge, given that only about 1 compound in 20 successfully emerges from an anti-infective drug discovery program. Since new drugs for TB should only be used in combination, to prevent resistance, it would be a responsible act of global leadership to take whatever steps are necessary to attract as many new lead compounds into the pipeline as quickly as possible.

Another possible approach for generating a significant scale-up of TB drug discovery is to improve public sector capacity for running drug discovery programs. Government funding agencies could establish a medicinal chemistry resource center that would work as a core facility offering free lead optimization and ADMET (absorption, distribution, metabolism, excretion, and toxicology) studies in animal models. Such a facility would be directed by scientists with experience in drug discovery, and could also carry out training activities in order to ensure medicinal chemistry expertise in academia. A major challenge, though, would be attracting talented scientists to the not-for-profit medicinal chemistry sector and retaining them in competition with industry. Moreover, to invest in medicinal chemistry for the public sector without the other technologies, resources, and expertise that go into drug development might result in limited success or strategic failure (Casenghi *et al.*, 2007).

CHAPTER 3
PHYTOCHEMISTRY AND MEDICINAL USES OF SELECTED MEDICINAL PLANTS

3.1. Introduction

Traditional medicine usually lacks scientific proof of efficacy, which does not necessarily mean that the medicines used are not valuable, but only that more scientific work is needed to investigate their validity (Mabogo, 1990). Low costs and accessibility are not the only advantages of using traditional healers and herbalists; there is also a strong belief in the efficacy of traditional medicine. Plants have long provided mankind with herbal remedies for many infectious diseases and even today, they continue to play a major role in primary health as therapeutic remedies in developing countries (Sokmen *et al*., 1999).

Plant-derived medicines have been part of traditional health care around the world for thousands of years, and there is an increasing interest in plants sources to fight microbial diseases. These plant drugs contain chemical compounds that act individually or in combination on the human body to prevent disorders and to maintain health (Palombo and Semple, 2001; van Wyk and Wink, 2004). Plants contain numerous biological active compounds, many of which have been shown to have antimicrobial activity (Lopez *et al*., 2001; Karaman *et al*., 2001). The search for biologically active extracts based on traditionally used plants is still relevant due to the appearance of microbial resistance to many antibiotics and the occurrence of fatal opportunistic infections. Ethnobotanical data has proved to be useful in the search of compounds isolated from plants (Penna *et al*., 2001). Thus, there has been a renewed interest in phytomedicine during the last decade and these days many medicinal plant species are being screened for pharmacological activities (Zakaria, 1991; Gautam *et al*., 2007).

3.2. Plants selected

Many modern medicines have their origins in plants that are often used in the treatment of illness and disease. Plants and their derivatives contribute to more than 50% of all medicine used worldwide. It is estimated that in South Africa there are 27 million people spending between 4 and 6% of their annual income on traditional medicine and services (Mander, 1999). Several works have been published recording the ethnobotanical use of plants in South Africa (Watt and Breyer-Brandwijk, 1962, Hutchings *et al*., 1995, van Wyk *et al*., 1997). The demand for medicinal plants is likely to remain high in the future. Urban consumers anticipate that their consumption of indigenous medicine will either remain at current levels or increase. This is despite the fact that indigenous medicine is more expensive than subsidised Western health care provided by the Government. There are a wide range of ailments and needs that cannot adequately be treated by Western medicine. This implies that indigenous medicine is basic consumer goods, often essential for the welfare of black households (Mander, 1999).

Many South African plants have medicinal usage for treatment of TB and related symptoms such as coughing, respiratory ailments and fever. Due to this diversity, 3 rural settlements (Manini, Nzhelele and Thengwe) in the Venda region of the Lipompo Province of South Africa were selected for obtaining information about the plants being used for TB-related symptoms. Prior to the interview, the traditional healers were explained the importance of the information they were providing and the type of research that was planned to conduct on the plants they will provide. They were also informed that the results and any profitable outcome would be communicated to them. This was done in order to safeguard the interests of both the parties. Three traditional healers and few local people agreed to be interviewed.

Symptoms of the various forms of TB infections were described to the traditional healers so as to enable them give the appropriate plants they use in the management of these conditions. Questions were as follows:

Traditional healer questionnaire

1. Which medicinal plants do you use to treat tuberculosis, TB-related symptoms such as for cough, chest pain, blood in the sputum, etc.

2. Who collects the medicinal plants for you?

3. Where do you collect these medicinal plants (the name of the place) and how often do you collect per month or per week?

4. What plant parts do you use?

5. How do you package the plant materials?

6. How long can you keep the medicinal plants before they loose their healing properties?

7. What do you use for preparing the medicinal plants?

8. Do these medicinal plants have any substitutes if they are not available?

9. Do you accept the substitutes as being effective?

10. Where do most of your patients come from?

11. Do your patients ask for the medicinal plants by name or do they describe their problem and ask you to prescribe the appropriate medicine?

12. Why do patients come to you instead of visiting clinics?

13. Do you think your patients are comfortable coming to a traditional healer?

14. What is the average cost of a treatment per patient?

15. Do you refer your patients to the Western doctors if TB-symptoms are serious?

Collection of the plant materials was permitted by the traditional healers in limited quantities. The scientific names, common names, plant parts used, voucher specimens, medicinal uses and the traditional usage of selected ethnobotanically South African plants are explained in Table 3.1. A detailed description of each selected medicinal plants for the present study is as follows:

3.2.1. *Artemisia afra* Jacq. ex Willd.

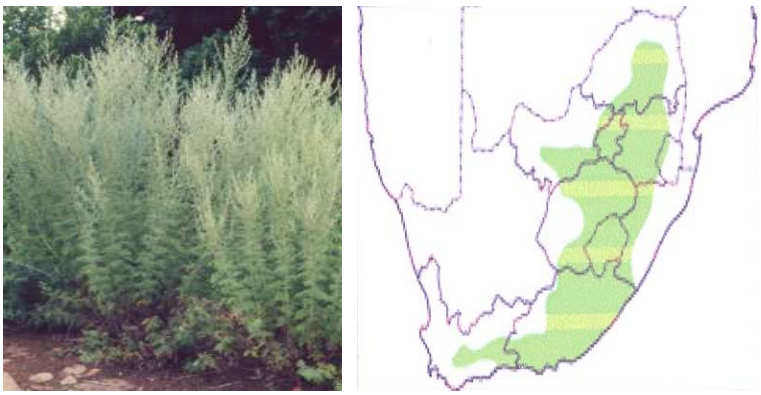


Figure 3.1. ***Artemisia afra*** **and distribution in South Africa (van Wyk *et al.*, 1997)**

3.2.1.1. Description and distribution

A. afra (Figure 3.1) named after the Greek goddess of hunting, *Artemis*, belongs to the family Asteraceae, and is commonly known as African wormwood (Jackson, 1990). The plant is a highly aromatic, multi-stemmed perennial shrub that grows into thick, bushy, slightly untidy clumps, usually with tall stems up to 2m high, but sometimes as low as 0.6m. The feathery leaves are finely divided and usually have a greyish-green colour. The pale yellow flowers are borne along the branch ends and are produced at the end of summer. The plant has an easily identifiable aromatic odour and smells pungent and sweet after bruising (Liu *et al.*, 2008). *A. afra* is a common species in South Africa with a wide distribution from the Cederberg Mountains in the Cape. It is also abundantly found in Kwazulu-Natal.

Table 3.1. Traditional uses of selected South African medicinal plants used for the present study

Scientific name (Family)	Common names (Venda local name)	Plant parts used	Medicinal use	Voucher specimen number
Atermisia afra Jacq. ex Willd. (Asteraceae)	Wild Wormwood (Chiba)	Roots	Colds, chest pains, fever, malaria	92824
Dodonaea angustifolia L.f. (Sapindaceae)	Sand olive (Tshikhopha)	Leaves	Cancer, fever, tuberculosis	93724
Drosera capensis L. (Droseraceae)	Cape sundew (Munzere)	Roots	Asthma, bronchitis, cancer, fever	84924
Galenia africana L. (Aizoaceae)	Yellow bush (Muferedonga)	Leaves	Asthma, chest pains, tuberculosis	93723
Prunus africana Hook. f. (Rosaceae)	Red stinkwood (Mulala-maanga)	Bark	Chest pains, fever, stomach disorders	71357
Syzygium cordatum Hochst. ex Krauss. (Myrtaceae)	Water berry (Mutu)	Bark	Coughs, diarrhoea, tuberculosis	95547
Ziziphus mucronata Willd. (Rhamnaceae)	Buffalo thorn (Mutshetshete)	Bark	Bronchitis, chest pains, fever	94270

province from the coast to the Drakensberg. *A. afra* also grows in northwards to tropical East Africa and stretching as far as northern Ethiopia (Figure 3.1; van Wyk *et al*., 1997; van Wyk and Wink, 2004).

3.2.1.2. Medicinal uses

In many parts of South Africa, this plant is traditionally used for a wide variety of ailments, including coughs, chest pains, sore throats, asthma, headaches, malaria and intestinal worms. *A. afra* is used in many different ways and one of the most common practices is to insert fresh leaves into the nostrils to clear blocked nasal passages (van Wyk *et al*., 1997). A syrup is prepared and used for bronchial troubles. Another, maybe not so common use, is to place leaves in socks for sweaty feet. The roots, stems and leaves are used in many different ways and are taken as enemas, poultices, infusions, body washes, lotions, smoked, snuffed or drunk as a tea. (Watt and Breyer-Brandwijk 1962). The use of other medicinal plants or substances in combination with *A. afra* is also documented. Preparations of *A. afra* in combination with *Eucalyptus globus* is employed to treat influenza. The infusion of the leaves and stems of *A. afra* with *Lippia asperifolia* is used as a formula for fevers, measles against lung inflammation (Watt and Breyer-Brandwijk, 1962).

3.2.1.3. Phytochemistry and biological activity

Phytochemical studies have proved the presence of scopoletin in the flowerheads (Bohlmann and Zdero, 1972), alkaloids, flavonoids, sterols, saponins, diterpenes, tannins and volatile oil in the leaves of *A. afra* (Silbernagel *et al*., 1990). Other studies have identified the triterpenes α- and β-amyrin, friedelin as well as alkanes ceryl cerotinate from the roots of this plant species. The volatile oil contains a large number of monoterpenes (1,8-cineole, α-thujone, camphor and β-borneol) and sesquiterpenes (davanone, chrysanthenyl acetate). The volatile oil obtained from aerial parts of *A. afra* showed antimicrobial activity against a range of bacteria, fungi and antioxidant activity in preventing decolouration of β–carotene and linoleic acid (Graven *et al*.,

1992). Coumarins such as scopoletin and isofraxidin have been isolated from *A. afra* (Bohlmann and Zdero, 1972). The coumarins are able to inhibit various enzymes such as β amylase, invertases and phenolases. The flavonol, axillarin was isolated from *A. afra*. Kraft *et al* (2003) discovered two types of compounds, flavonoids (7-methoxyacacetin, acetin, genkwanin and apigenin) and lactones responsible for antiplasmodial activity. Terpenoids and acetylenes have also been reported to have been isolated from *A. afra* for antibacterial activity (Wollenweber *et al* 1989; Jakupovic *et al*., 1988).

3.2.2. *Dodonaea angustifolia* L. f.

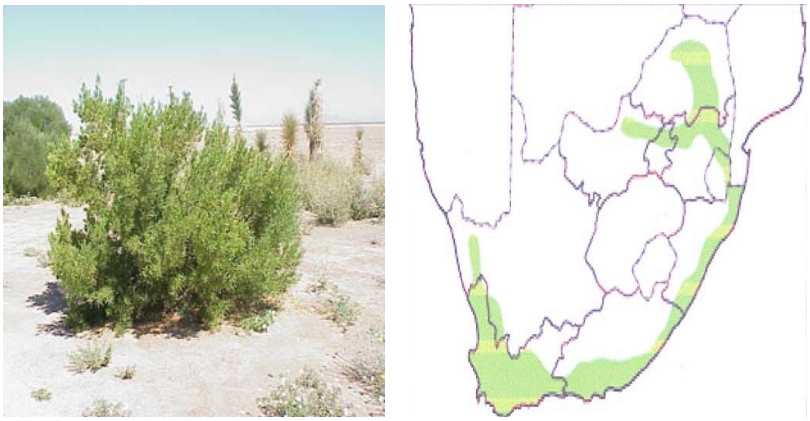


Figure 3.2. ***Dodonaea angustifolia*** **and distribution in South Africa (van Wyk *et al*., 1997)**

3.2.2.1. Description and distribution

Dodonaea angustifolia (Figure 3.2) belongs to the family Sapindaceae, and is commonly known as sand olive. The plant is a shrub about 5m in height, occurring in a wide range of habitats, from deserts to forest margins. The long, narrow leaves are pale green and shiny and the flowers are yellowish-green. *Dodonaea angustifolia* is widely distributed from Namaqwaland to Cape Peninsula and eastwards to Port Elizabeth and also along the coastal belt north into Zimbabwe and Mozambique (Figure 3.2; van Wyk *et al*., 1997).

3.2.2.2. Medicinal uses

The leaves and roots of this plant are used for arthritis, cancer, chest complains, fever, influenza, measles, pneumonia, stomach disorders, TB, throat infections, skin rashes and hypertension (Palmer and Pitman, 1972; van Wyk and Gericke, 2000; van Wyk *et al*., 1997). In the early Cape times, a decoction of the leaves, was used as a remedy for sore throats and fever (van Wyk *et al*., 1997).

3.2.2.3. Phytochemistry and biological activity

Dodonic acid, diterpenoids, glycosides and hautriwaic acid were isolated from leaves of several *Dodonaea* species. The acids are used as essential oils (Sachdev and Kulshreshtha, 1984). Quinones, tannins and saponins were also detected in preliminary tests (Ibid, 1983). Flavones (santin, penduletin and aliarin), flavonoids, flavanones (pinocembrin) and a new flavonoid (5,7,4′-trihydroxy-3,6-dimethoxyflavone) were isolated from the roots of this plant and were reported to have antioxidant, antifungal and α–amylase activities. Water extracts of *Dodonaea angustifolia* indicated the analgesic and antipyretic effects (Amabeoku *et al*., 2001). Other *Dodonaea* species have been investigated for anti-inflammatory, antifungal and antibacterial activity (van Wyk *et al*., 1997).

3.2.3. *Drosera capensis* L.

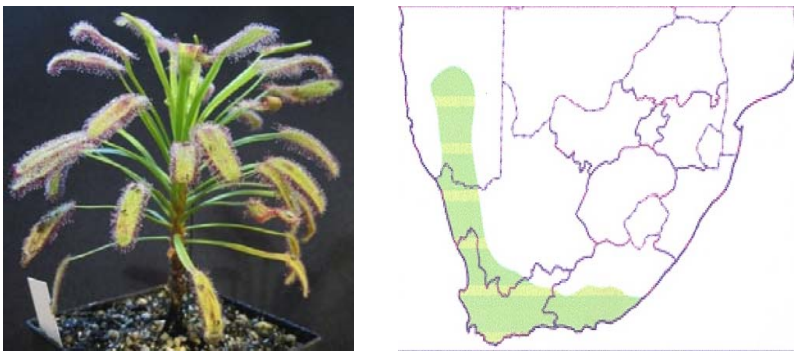


Figure 3.3. ***Drosera capensis*** **and distribution in South Africa (Gibson, 1993)**

3.2.3.1. Description and distribution

Drosera capensis (Figure 3.3) is a carnivorous plant belonging to the family Droseraceae, and is commonly known as Cape sundew. The plant is a perennial herb about 30cm height, naturally in the Cape region of South Africa (Figure 3.3; Gibson, 1993), and can be found in marshes, along streams, permanent seeps or damp areas of fynbos. *Drosera capensis* produces strap-like leaves, up to 15cm long and 1cm wide. The plant has a tendency to retain the dead leaves of previous seasons, and the main stem of the plant can become quite long and woody with time. When insects are first trapped, the leaves roll lengthwise by thigmotropism, and this aids digestion by bringing more digestive glands in contact with the prey item (Correa *et al*., 2005; Schlauer, 1996).

3.2.3.2. Medicinal uses

Drosera capensis is recommended by herbalists for treatment of stomach ulcers, bronchitis, whooping coughs, and asthma. Extracts of the leaves are used externally for warts, corns and sunburn. Disorders such as TB, coughs, eye and ear infection, liver pain, morning sickness, stomach conditions, syphilis, toothache and intestinal problems are treated internally with extracts made from the leaves of this plant.

3.2.3.3. Phytochemistry and biological activity

The flowering parts of the plant were found to be antibacterial (van Wyk *et al*., 1997). Compounds such as flavonoids (kaempferol, myricetin, quercetin and hyperoxide) known to have antioxidant activities **were** isolated from *D. capensis* (Marczak *et al*., 2005). Rossoliside (7–methyl–hydrojuglone–4–glucoside) and other constituents such as carotenoids, plant acids (citric acid, formic acid, gallic acid and malic acid), resins, tannins and ascorbic acid (vitamin C) all of which have been found to have antimicrobial activities were isolated earlier by Crouch *et al*., 1990. Quinones (plumbagin and hydroplumbagin glucoside) purified from the aerial parts of the plant showed TB and HIV inhibitory activities. The major compound in *D. capensis*,

plumbagin, has been used in bronchial treatments, particularly whooping cough. Plumbagin was also found to have an inhibitory action on the growth of the TB bacterium (Heise and Steenken, 1941). Kubo *et al*, 1980, reported that plumbagin exhibited relatively specific antimicrobial activity against yeasts and is also potent insect antifeedant against the larvae of African army worms. Anti-spasmodic agents have been found by scientists in some *Drosera* species (Correa *et al*., 2005).

3.2.4. *Galenia africana* L. var. *africana*

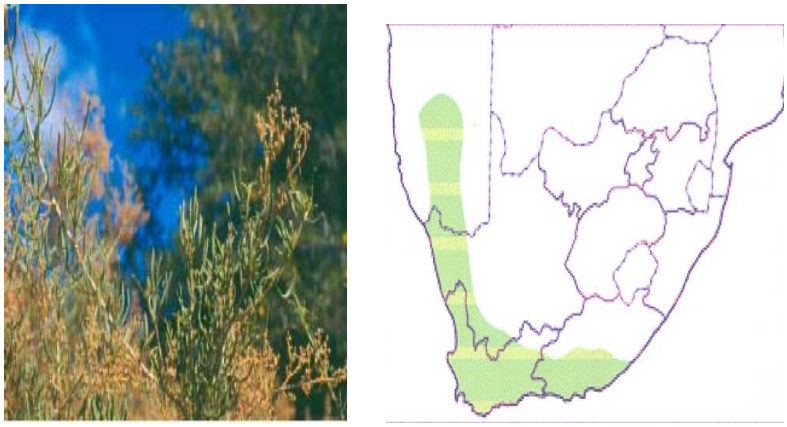


Figure 3.4. ***Galenia africana*** **and distribution in South Africa (van Wyk *et al*., 1997).**

3.2.4.1. Description and distribution

G. africana (Figure 3.4) belongs to the family Aizoaceae and is commonly known as yellow bush. It is an aromatic, woody perennial shrub growing at a height of 0.5 – 1.5m. The plant is distributed on dry flats and lower slopes from the Northern Cape, Namaqwaland to the Karoo and to the Eastern Cape (Figure 3.4). The stems are pale coloured and the leaves bright green when young and turning yellow when older (van Wyk *et al*., 1997).

3.2.4.2. Medicinal uses

G. africana is used to treat venereal sores, asthma, coughs, wounds, eye infections, TB and skin diseases. Indigenous tribes chew the leaves to relieve toothache and a decoction is used as a lotion for skin diseases such as ringworm and to relieve inflammation of the eyes (Vries *et al*., 2005; Watt and Breyer-Brandwijk, 1962).

3.2.4.3. Phytochemistry and biological activity

Preliminary chemical tests demonstrated the presence of alkaloids but not of saponins, tannins and reducing sugars (Vries *et al*., 2005). There is no further information regarding the biological activity of other secondary metabolites isolated from *G. africana*.

3.2.5. *Prunus africana* Hook. f.

Figure 3.5. ***Prunus africana*** **and distribution in South Africa (van Wyk *et al*., 1997).**

3.2.5.1. Description and distribution

P. africana (Figure 3.5) belongs to the family Rosaceae, and is commonly known as red stinkwood. *P. africana* is confined to evergreen forests from near the coast to the mist belt and montane forests in KwaZulu-Natal, Eastern Cape, Swaziland,

Mpumalanga, Zimbabwe and Sub-Saharan African regions (Figure 3.5; van Wyk *et al*., 1997; van Wyk and Wink, 2004).

3.2.5.2. Medicinal uses

The bark of *P. africana* is used as an effective drug for chest pain, diarrhoea and fever. The extract of powdered bark is traditionally drunk as tea for genito-urinary complaints, allergies, inflammation, kidney diseases, malaria and fever (Pujol, 1990). In South Africa the bark is used to treat chest pains. The bark extract has become popular in Europe for the treatment of benign prostate hypertrophy. It is also reputed to be very poisonous and to have magical properties (van Wyk *et al*., 1997).

3.2.5.3. Phytochemistry and biological activity

Phytosterols (free glycosylated β-sitostenone, daucosterol and campesterol) have been isolated from the bark extracts and are used as anti-inflammatory, phytosterols are also reported to inhibit cellular increase, lower cholesterol, regulate the immune system and treat benign prostatic hyperplasia (BPH). The sterols and lipids of *P. africana* may act by preventing the conversion of testosterone to dihydrotestosterone (DHT), aromatose inhibition (prevention of conversion of DHT to androstandiol and hence blocking the synthesis of oestrogen), blocking of leukotriene synthesis by inhibition of 5 lipoxygenase and also reduction in oedema by inhibition of glucosyl transferase and β glucoronidase (Urbeti *et al*., 1990).

The presence of amygdalin and a cyanogenic glycoside, used for inflammation, kidney diseases, malaria and fever, has been reported from bark and leaves extracts (van Wyk, 2004). *P. africana* also contains pentacyclic triterpenoid esters, linear aliphatic alcohol and their ferulic acid esters (van Wyk *et al*., 1997). Reduction in oedema and inflammation as well as diminished histamine-induced vessel permeability has been demonstrated by the presence of ferulic acid esters for hypocholesterolaemic and phytosterols for anti-inflammatory (Awang, 1997).

3.2.6. *Syzygium cordatum* Hochst. ex Krauss.

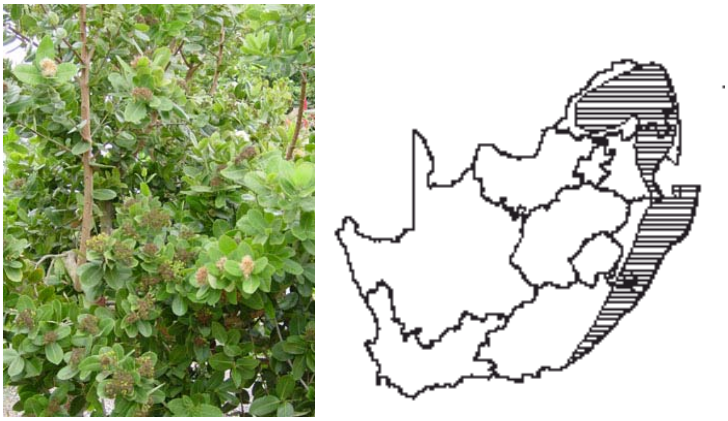


Figure 3.6. *Syzygium cordatum* and distribution in South Africa (van Wyk *et al*., 1997).

3.2.6.1. Description and distribution

S. cordatum (Figure 3.6) belongs to the family Myrtaceae, and is commonly known as water berry. *S. cordatum* is a medium-sized tree up to 15m in height, with a rough dark brown bark. The leaves are broad, circular with a bluish-green colour. The tree is commonly found near streams, forest margins or in swampy spots. *S. cordatum* has a wide distribution occurring in the eastern and north-eastern parts of South Africa, along stream banks from Kwazulu-Natal northwards to Mozambique (Figure 3.6; van Wyk *et al*., 1997).

3.2.6.2. Medicinal uses

S. cordatum is used to treat respiratory ailments and TB. In central Africa the tree is known as a remedy for stomach ache, wounds and diarrhoea. It is also used as an emetic (van Wyk *et al*., 1997). *S. cordatum* leaf extract contains compounds that could be effective in mild diabetes mellitus or in cases of glucose tolerance impairment (Musabayane *et al*., 2005). Methanol and water extracts of *S. cordatum* have been found to have antifungal activity against *Candida albicans* (Steenkamp *et*

*a*l., 2007).

3.2.6.3. Phytochemistry and biological activity

Previous studies showed that wood and bark extracts of *S. cordatum* contain triterpenoids (arjunolic acid, friedelin and epi-friedelinol), glucose, tannins, ellagic acid (hexahydroxydiphenic acid) and a gallic acid-ellagic acid complex for antidermatophytic activity (Candy *et al*., 1968). The bark also contains proanthocyanidins for gastroprotective effect (van Wyk *et al*., 1997). Leucodelphinidin and leucocyanidin were detected in bark and leaf and have been found to have antibacterial activity (Candy *et al*., 1968). Presence of triterpene (betulinic acid, oleanolic acid, a mixture of 2-hydroxyoleanolic acid, 2-hydroxyursolic acid, arjunolic acid, asiatic acid, a mixture of terminolic acid, 6-hydroxyasiatic acid, and a mixture of arjunolic acid) and triterpenoids showed antibacterial and antidermatophytic properties (Djoukeng *et al*., 2005). Ndhlala *et al* (2007) found more phenolics and flavonols from the peels of *S. cordatum* fruits. Essential oils and phenolic compounds (catechin and ferulic acid) from other *Syzygium* species (*S. cumini* and *S. travancoricum*) have been found to have antibacterial and antioxidant activity (Ruan *et al*., 2008, Shafi *et al*., 2002).

3.2.7. *Ziziphus mucronata* Willd.

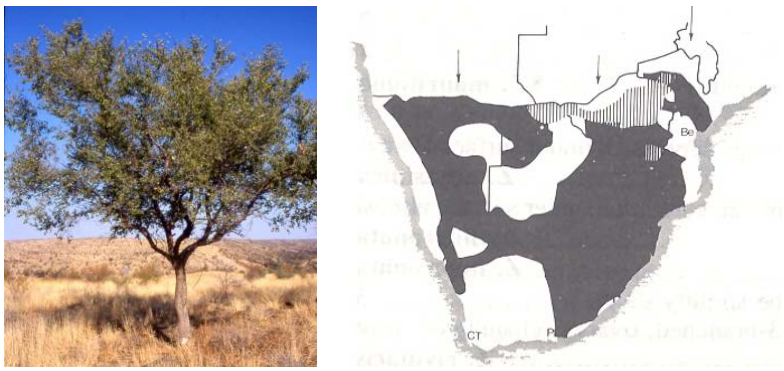


Figure 3.7. ***Ziziphus mucronata*** **and distribution in South Africa (van Wyk *et al*., 1997)**

3.2.7.1. Description and distribution

Z. mucronata (Figure 3.7) belongs to the family Rhamnaceae, and is commonly known as buffalo-thorn. *Z. mucronata* is a small to medium-sized tree up to 15m in height. The plant has strong paired spines of which one is straight and the other curved. The bark is dark grey-brown and only smooth when young. The leaves are hairless, shiny and alternate. Flowers are very small, yellow-green and crowded into heads. Fruits are round (~ 2cm), dark red-brown when ripe and arranged in stalked bunches. The plant is widespread; from southern Africa to Arabia in the north (Figure 3.7; van Wyk *et al*., 1997).

3.2.7.2. Medicinal uses

This tree is used extensively in traditional African medicine and some of the uses are so common among different cultural groups. In East Africa, roots are used for treating snake bites (Hutchings *et al.* 1996). Others use a branch round the village to protect it from evil spirits, as it is believed to keep evil spirits away. Zulus and Swazis use the buffalo thorn in connection with burial rites. Various tribes use infusions or pastes of the roots and leaves internally and externally for boils, stomach ailments, skin ulcers, carbuncles and swollen glands. A poultice of meal made with a root decoction or powdered baked root is widely used to treat pain of all kinds (Palmer and Pitman, 1972). Bark infusions are used as expectorants in coughs and chest pains, decoctions of the roots and leaves are applied externally to boils, sores and glandular swellings for the relief pain (van Wyk *et al*., 1997).

3.2.7.3. Phytochemistry and biological activity

Several alkaloids, referred to as peptide alkaloids are known to accumulate in *Z. mucronata* and have been reported to have anthelmintic activity. A new cyclopeptide alkaloid (mucronine J) was isolated together with previously known alkaloids (abyssenine A and mucronine D) from the dichloromethane root extract of *Z. mucronata* (Auvin *et al*., 1996). Frangufoline (sanjonine A) occurs in the seeds which

showed antibacterial activity (Dimitris *et al*., 1985). The fruit contains tetracyclic triterpenoid saponins and flavonoids which exhibited antifungal activity (van Wyk and Wink, 2004). *Z. mucronata* has been found to have antisickling activity due to the presence of anthocyanins (Mpiana *et al*., 2008).

3.3. Discussion and Conclusion

Many modern medicines have originated from plants that are often used in the treatment of illness and disease. People on all continents have since prehistoric times used hundreds to thousands of their indigenous plants for the treatment of ailments. In fact, plants and their derivatives contribute to more than 50% of all medicine used worldwide. In this way traditional healers and their medicines played an important role in developing western medicines. Herbal medicine is a major component in all traditional medicine systems, and a common element in ayurvedic, homeopathic, naturopathic, traditional Chinese and Native American medicines. WHO estimates that 80% of the world's population presently uses herbal medicine for some aspect of primary health care (WHO, 2003). The search for drugs and dietary supplements derived from plants has accelerated in recent years.

Ethnopharmacologists, microbiologists, botanists and natural-product chemists are combing the Earth for phytochemicals and leads that could be developed for the treatment of infectious diseases such as TB (Cowan, 1999). The traditional herbal healer therapies contain many medicines for one ailment. Out of the various medicines, one is selected by the herbal healer against a particular disease according to the symptoms and secondary effects. Several plants are identified and used against one disease and are used according to their availability in the region. The different preparations of plant parts are prepared and used by the traditional medicinal man as a cure for a particular disease. In this study, we selected some of the medicinal plants that are used for respiratory ailments such as asthma, colds, chest pains, fever and TB. Information was culled from traditional healers and people who use some of these plants against respiratory tract diseases.

CHAPTER 4

ANTITUBERCULOSIS ACTIVITY OF SELECTED MEDICINAL PLANTS AGAINST *MYCOBACTERIUM SMEGMATIS* AND *M. TUBERCULOSIS*

4.1. Introduction

Plants have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. One of the major issues when developing new drugs from plants is to examine the uses claimed in traditional use. Many reports on the pharmacological testing of crude extracts have been published by investigators (Farnsworth *et al*., 1985). Traditional medicine has served as a source of alternative medicine, new pharmaceuticals and healthcare products. Medicinal plants are important for pharmacological research and drug development, not only when the plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). The screening of plant extracts is of great interest to scientists for the possible treatment of many diseases (Dimayuga and Garcia, 1991).

The TB crisis is likely to escalate since the human immunodeficiency virus (HIV) epidemic has triggered an even greater increase in the number of TB cases. The majority of TB patients are aged between 15 to 45 years, persons in their most productive years of life (Girling, 1989). The development of drug resistance, as well as the appearance of side effects of certain drugs, has led to numerous studies to validate the traditional use of antitubercular medicinal plants by investigating the biological activity of extracts of these medicinal plants (Newton *et al*., 2000). The aim of the present study was to evaluate selected South African medicinal plants used traditionally to treat TB or related symptoms, such as coughs, fever, chest pains, lung infections and other respiratory tract diseases, against *Mycobacterium smegmatis* and

M. tuberculosis.

4.2. Materials and Methods

4.2.1. Plant materials

The selection of plant species for this study was based on their traditional use for TB, the information being culled from published sources and traditional healers (Chapter 3, Table 3.1). Plants and the parts used were: *Artemisia afra*, Jacq. ex Willd (leaves), *Dodonea angustifolia* L. f. (leaves), *Drosera capensis* L. (leaves), *Galenia africana* L.var. *africana* (leaves), *Prunus africana* Hook. f. (bark), *Syzygium cordatum* Hochst. ex Krauss (bark) and *Ziziphus mucronata* Willd (bark). Voucher specimens were identified and deposited at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa (Chapter 3, Table 3.1). Different plant parts (bark, leaves and roots) of the seven selected plant species, representing different families, were collected in autumn from different areas in South Africa. Approximately 3.0 kg of fresh plant material of each species was air-dried and ground.

4.2.2. Preparation of plant extracts

The extraction yield of the extracts from plant species highly depend on the solvent polarity, which determines both qualitatively and quantitatively the extracted compounds. The highest yields are usually achieved with ethanol, methanol and their mixtures with water, although other solvents (acetone or ethyl acetate) have been widely used for the extraction of polyphenols from plants. Ethanol and water are the most widely used solvents because of their low toxicity and high extraction yield with the advantage of modulating the polarity of the solvent by using ethanol / water mixtures at different ratios (Sineiro *et al*., 2008).

In the present study only ethanol was selected because of its similarity with water, especially with regard to polarity. Hundred and fifty grams of each plant part were

extracted with two successive 500.0 mL portions of ethanol for 24 hours at room temperature. The extracts were concentrated to dryness at reduced pressure with a rotary evaporator at 40^{o}C.

4.2.3. Microorganisms

The microorganisms, *M. smegmatis* (MC^{2} 155) and a drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) were obtained from American Type, MD, USA Culture Collection. *M. smegmatis* was cultured onto Middlebrook 7H11 agar base (7H11) and allowed to grow for 24 hours at 37^{0}C. *M. tuberculosis* was plated onto Löwenstein-Jensen medium and allowed to grow for 3 - 4 weeks at 37^{o}C.

4.2.3.1. Antimycobacterial activity on *M. smegmatis* using the agar method

All the ethanol plant extracts were dissolved in 10% dimethyl sulphoxide (DMSO) in sterile Middlebrook 7H9 broth (Sigma-Aldrich, South Africa), to obtain a concentration of 500.0 mg/mL. The bacteria were carefully scraped and transferred into a sterilized glass tube containing a few glass beads (2 mm in diameter) and 50.0 mL of 7H9 broth base was added to the culture and then recovered for testing by growth in 7H9 broth base for 24 hours at 37^{o}C.

Before streaking, the culture was adjusted to an optical density (OD) of 0.2 log-phase (an optical density value which would ensure that the bacteria was at the start of the log phase when the test commenced) at 550 nm using spectrophotometer, yielding 1.26 x 10^{8} colony-forming units per millilitre (CFU/mL) (Salie *et al*., 1996; Newton *et al*., 2002).

The MIC of the ethanol extracts was determined by incorporating various amounts (5.0 – 200.0 mg/mL) of each into petri dishes containing the culture media. Before congealing, 5.0 mL of 7H11 agar medium containing the plant extract was added aseptically to each petri dish and swirled carefully until the agar solidified. The bacteria was streaked in radial patterns on the 7H11 agar plates containing the plant

extracts, before incubating at 37°C for 24 hours (Mitscher *et al*., 1972). All extracts were tested at 200.0, 100.0, 50.0, 25.0, 10.0 and 5.0 mg/mL. Ciprofloxacin (Sigma-Aldrich, South Africa) added to 7H11 agar medium at final concentrations of 0.5, 0.01 and 0.05 mg/mL served as positive control. Three blank plates containing only 7H11 agar medium and three with 10% DMSO without plant extracts served as negative controls. The MIC was regarded as the lowest concentration of the extracts that did not permit any visible growth of *M. smegmatis*. Tests were done in triplicates.

4.2.3.2. Microplate susceptibility testing against *M. smegmatis*

All extracts were tested against *M. smegmatis* using the microplate dilution method (Newton *et al*., 2002). The MIC and the bacterial effect (minimum bactericidal concentration, MBC) were determined according to the methods described by Salie *et al*., 1996. The ethanol extracts were dissolved in 10% DMSO in sterile Middlebrook 7H9 broth base to obtain a stock concentration of 100.0 mg/mL. Serial two-fold dilutions of each sample to be evaluated were made with 7H9 broth to yield volumes of 100.0 µL/well with final concentrations ranging from 6.25 to 0.09 mg/mL. Ciprofloxacin served as the positive drug control. 100.0 µL of *M. smegmatis* suspension (0.2 log-phase, yielding 1.26 x 10^8 CFU/mL) was also added to each well containing the samples and mixed thoroughly to give a final volume of 200.0 µL/well. The solvent control DMSO at 2.5% v/v or less, in each well did not show inhibitory effects on the growth of *M. smegmatis*. Tests were done in triplicate.

The cultured microplates were sealed with parafilm and incubated at 37^{0}C for 24 hours. The MIC of the samples was detected following the addition (40.0 µL) of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT, Sigma-Aldrich, South Africa) and incubated at 37^{0}C for 30 minutes (Eloff, 1998). Viable bacteria reduced the yellow dye to a pink colour. The MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth. The MBC was determined by adding 50.0 µL aliquots of the preparations to 150.0 µL of 7H9 broth in all the wells. These preparations were incubated at 37°C for 48 hours.

The MBC was regarded as the lowest concentration of extract which did not produce an absorbance at 550 nm using an ELISA plate reader (Salie *et al*., 1996).

4.2.3.3. BACTEC: radiometric assay

The introduction of radiometric techniques in the field of mycobacteriology is a relatively recent development. Radiometric respiratory with the BACTEC TB-460 system (Becton Dickinson Diagnostic Instrument, Sparks, md; Figure 4.1), is a well-documented technique for testing susceptibility of *M. tuberculosis* and has been used as described previously by other researchers such as Lall and Meyer, 1999; Bapela 2005; and Mativandlela *et al*., 2006. The automated radiometric detection of *Mycobacterium* growth has opened new opportunities for quantitatively determination of the susceptibility testing of *Mycobacterium* on the basis of the MIC of the drugs.

The BACTEC procedure for drug susceptibility testing for *Mycobacterium* is based on the same basic principle employed in the conventional method, however, there are some differences. In the BACTEC radiometric assay a liquid medium (7H12) is used, and instead of counting colonies after about three weeks, the growth can be monitored radiometrically and the results reported within 5 to 6 days. On the other hand, in the conventional method, the growth of *M. tuberculosis* is monitored on a solid agar medium and the *Mycobacterium* colonies are counted only after about 3 weeks therefore the results are only available within 3 to 4 weeks.

Several published studies have reported that results obtained by the BACTEC method compared well with the conventional method (employing 7H10 / 7H11 media). The accuracy and reproducibility of the BACTEC method has also been evaluated with excellent results (Siddiqi *et al*., 1981; Snider *et al*., 1981). In the BACTEC method, drugs or the plant extracts are incorporated in a 7H12 Middlebrook TB medium, and the critical proportion of resistance of *M. tuberculosis* evaluated at the 1% level (Middlebrook *et al*., 1977).

Figure 4.1. BACTEC TB- 460 instrument

4.2.3.4. Antitubercular rapid radiometric assay using *M. tuberculosis*

A sensitive strain of *M. tuberculosis*, the $H37R_v$ references strain, was used in the screening procedure. A standard inoculum was prepared for the sensitive strain in Middlebrook – Dubos 7H9 broth containing 0,5% Tween 80 to obtain a concentration of 1.0 mg/mL (wet mass). The bacterial cultures, which were used to prepare the standard inoculum, were maintained on Lowenstein-Jensen medium. A representative amount of growth was taken from the cultures by using a sterile applicator stick. This sample was transferred to a sterile 16 x 125 mm screw capped round tube containing six to eight glass beads (1 – 2 mm) and 3.0 – 4.0 mL of the diluting fluid (0,1% Tween). A homogenous suspension was obtained by placing the tube on the Vortex mixer for five minutes and then left for 15 minutes to allow the particles to settle. After the large particles had settled, the supernatant, a homogeneous suspension was transferred into a separate sterile test tube and more Tween was added and adjusted approximately to McFarland no 1 turbidity standard (Youmans and Youmans, 1948).

Solutions of all the extracts were prepared in DMSO to obtain a stock concentration

of 500.0 mg/mL. Control experiments showed that a final concentration of DMSO (1%) in the medium had no adverse effect on the growth of *M. tuberculosis*. Isoniazid (INH; Sigma-Aldrich, South Africa) at a final concentration of 0.2 µg/mL served as the drug-control in our bioassay. All the extracts were tested at concentrations ranging from 5.0 to 0.1 mg/mL. A homogenous culture (0.1 mL of *M. tuberculosis*, yielding 1 x 10^4 to 1 x 10^5 CFU/mL), was inoculated into the vials containing the extracts, as well as in the control vials (Heifets *et al*., 1985). Three extract-free vials were used as controls (medium + 1% DMSO), two vials (V1) were inoculated in the same way as the vials containing the extracts, and one (V2) was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population (1 x 10^2 to 1 x 10^3 CFU/mL). The MIC was defined as the lowest concentration of the extract that inhibited > 99% of the bacterial population. When *Mycobacterium* grows in 7H12 medium containing a ^{14}C-labelled substrate (palmitic acid), they use the substrate and $^{14}CO_2$ is produced. The amount of $^{14}CO_2$ detected reflects the rate and amount of growth occurring in the sealed vial, and is expressed in terms of the growth index (GI; Middlebrook *et al*., 1977). Inoculated bottles were incubated at 37^0C and each bottle was assayed at 24 hours intervals at about the same time until cumulative results were interpretable to measure the GI.

The difference in the GI values of the last two days was designated as ΔGI. The GI readings of the vials containing the test extracts were compared with the control vials (V2). Readings were taken until the control vials, containing a 100 times lower dilution of the inoculum than the test vials, reached a GI of 30 or more. If the ΔGI values of the vials containing the test extracts were less than the control vials, the population was reported to be susceptible to the compound and if it was equal to or greater than that in the control vials, the test organisms were considered to be resistant to the drugs. Each test was replicated three times.

The bactericidal effect of the extracts that showed activity in the BACTEC system was assessed by plating the bacterial suspensions from individual BACTEC vials at the end of the experiment on 7H11 agar medium for viable count enumeration (Rastogi *et al*., 1991). A total of 0.1 mL of *M. tuberculosis* from BACTEC vials was

successively diluted 10-fold in sterile double-distilled water to give dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A total of 0.1 mL of 10^{-2} and 10^{-4} was plated onto 7H11 agar medium, and the resulting bacterial counts were enumerated after 20 days of incubation at 37^{0}C. The MBC was defined as the minimal concentration which effectively reduced, by at least 99%, the viable counts in the extract or compound-containing sample as compared with those in the control vials (extract or compound free vials).

All procedures involving the transfer of cultures were carried out in a biological safety cabinet and the bottle tops were wiped with gauze pads soaked with 5% phenol before removal from the hood. An ultraviolet light located under the hood of the BACTEC instrument could be turned on in case of an accident during the operation. In addition, a constant-volume air pump exhausted the chamber through absolute filters at a flow of 1 ft^3/min to protect the environment from aerosols that might be produced during collection of the gas sample from the inoculated bottles.

Whenever results suggested contamination (e.g., large, rapid increase in GI), the bottles were inspected and the organisms were stained by Ziehl-Neelsen stain to determine whether the visible microbial growth was a *Mycobacterium* (Kleeberg *et al*., 1980; WHO/TB/1998; Figure 4.2). With this stain, the bacilli appear as brilliantly stained red rods against a deep sky-blue background. The organisms often have a beaded appearance because of their polyphosphate content and unstained vacuoles (Joklik *et al*., 1968).

The basic reagents of Ziehl-Neelsen stain, and the staining procedures are as follows:

- **Ziehl-Neelsen carbolfuchin**

Fuchin

Basic fuchin 3.0 g

95% ethanol 100 ml

Basic fuchin was dissolved in ethanol .....Solution 1

Phenol

Phenol crystals 5.0 g

Distilled water 100 ml

Phenol crystals were dissolved in distilled water.....Solution 2

Working solution

10 ml of solution 1 was combined with 90 ml of solution 2.

- **Decolourising agent: 3% acid-alcohol**

Concentrated hydrochloric acid 3 ml

95% ethanol 97 ml

Concentrated hydrochloric acid was carefully added to 95% ethanol.

- **Counterstain: Methylene blue**

Methylene blue chloride 0.3 g

Distilled water 100 ml

Methylene blue chloride was dissolved in distilled water.

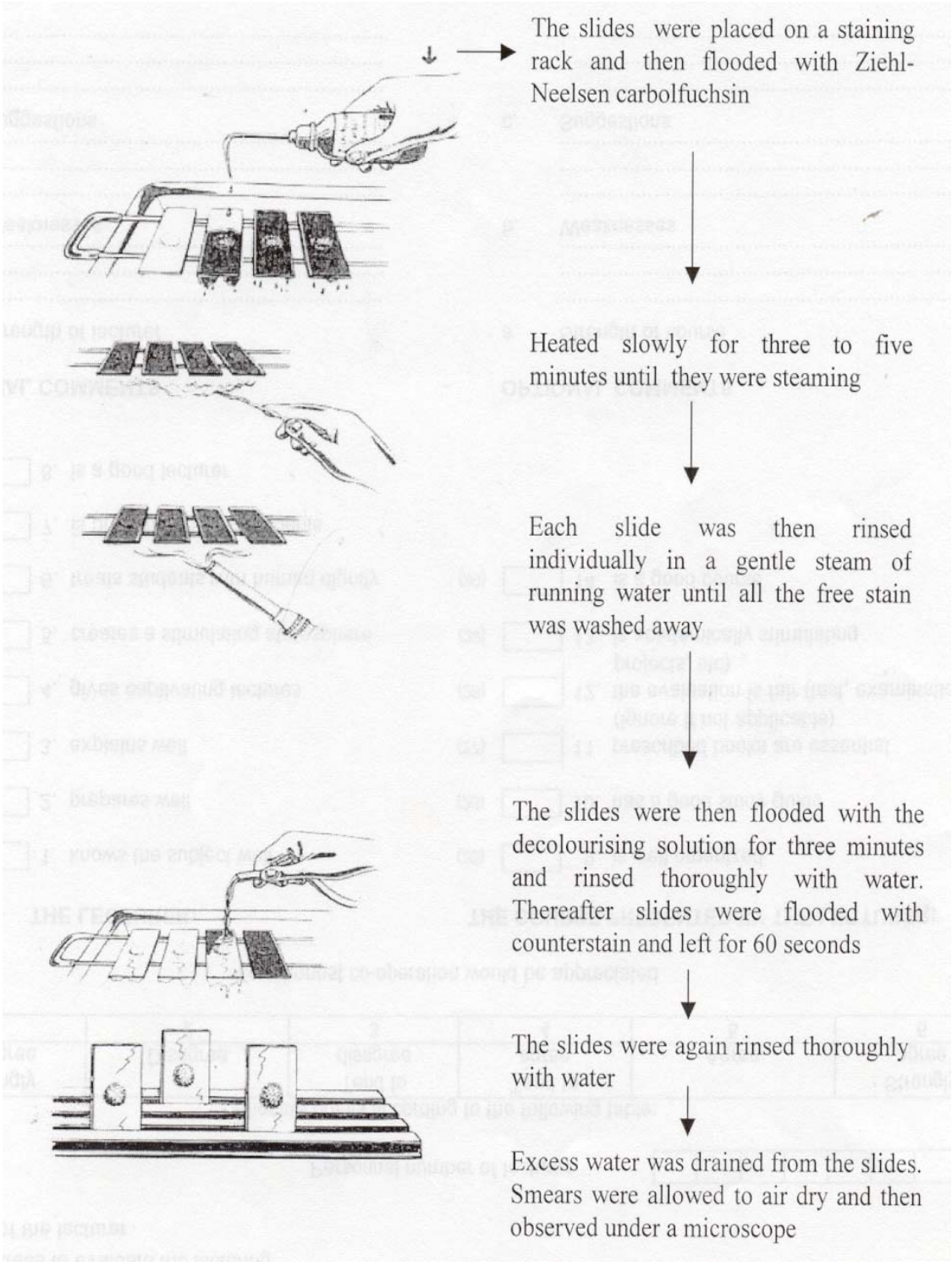


Figure 4.2. Ziehl-Neelsen staining (Kleeberg *et al*. 1980, WHO/ TB/97.258)

4.3. Results

Percentage yield (%w/w) of each ethanol extracts were: *A. afra* (12.4 dry wt), *Dodonaea angustifolia* (18.7 dry wt), *Drosera capensis* (10.9 dry wt), *G. africana* (24.8 dry wt), *P. africana* (21.5 dry wt), *S. cordatum* (32.3 dry wt) and *Z. mucronata* (27.5 dry wt) (Figure 4.3).

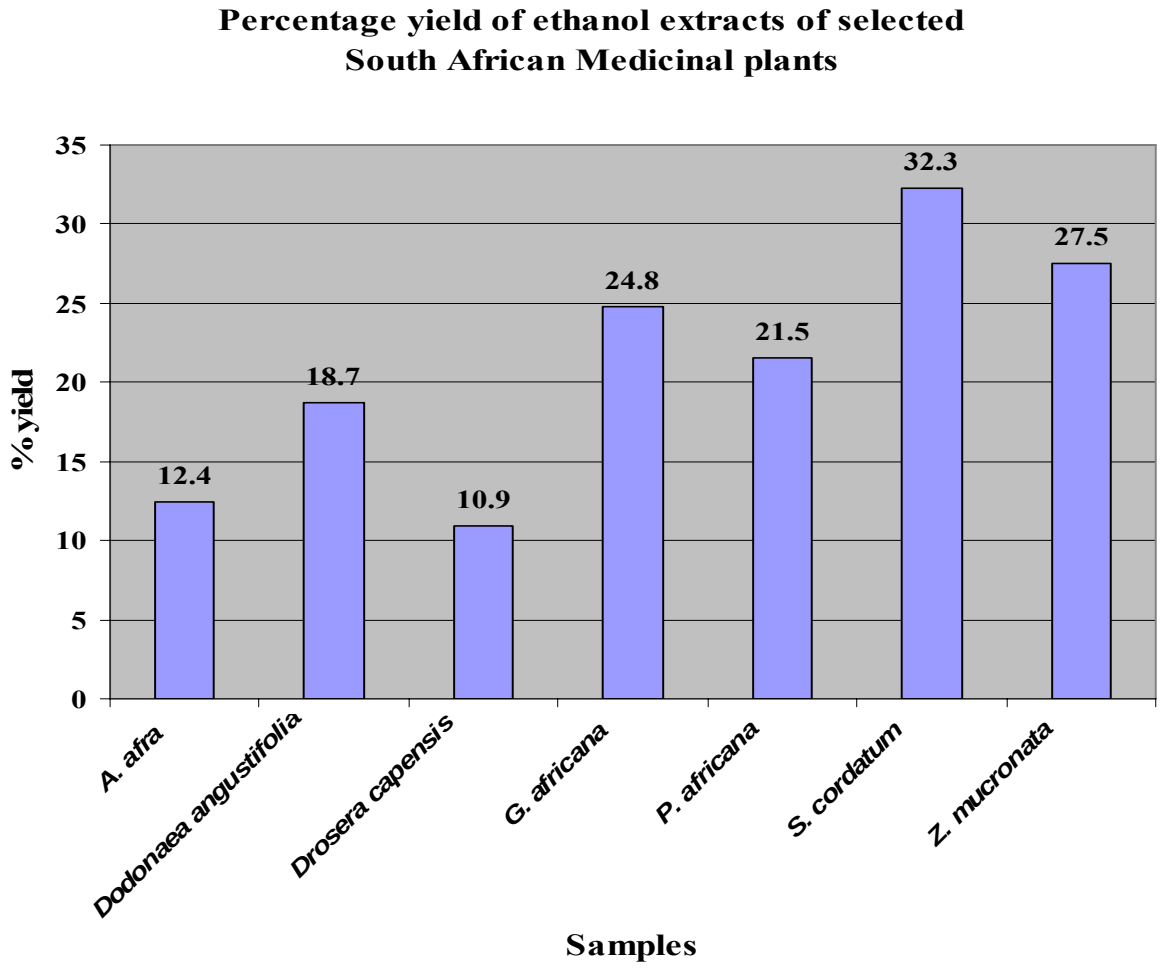


Figure 4.3. Percentage yield of ethanol crude plant extracts

4.3.1. Antituberculosis activity using the agar plate method

The antimycobacterial activity on agar plates showed that the ethanol extract of *G. africana* was active better than that of the other plant ethanol extracts (Figure 4.4).

The extract inhibited *M. smegmatis* at 50.0 mg/mL and this was followed by *Drosera capensis*, *Dodonaea angustifolia* and *A. afra* at a concentration of 100.0 mg/mL (Figure 4.5), whereas the other plant extracts (*P. africana*, *S. cordatum* and *Z. mucronata*) did not inhibit the growth of the bacteria tested. The reference antibiotic, ciprofloxacin inhibited the growth of *M. smegmatis* at 0.01 mg/mL (Table 4.1).

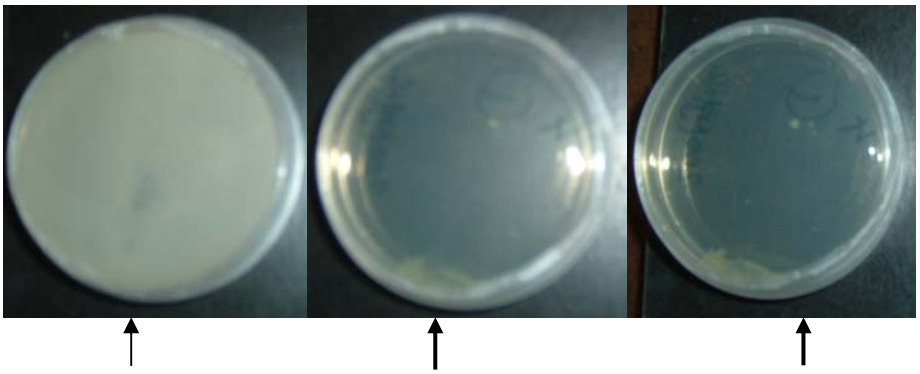


Figure 4.4. Antimycobacterial activity of ethanol extract of *G. africana* against *M. smegmatis* using the agar plate method at 50.0 mg/mL

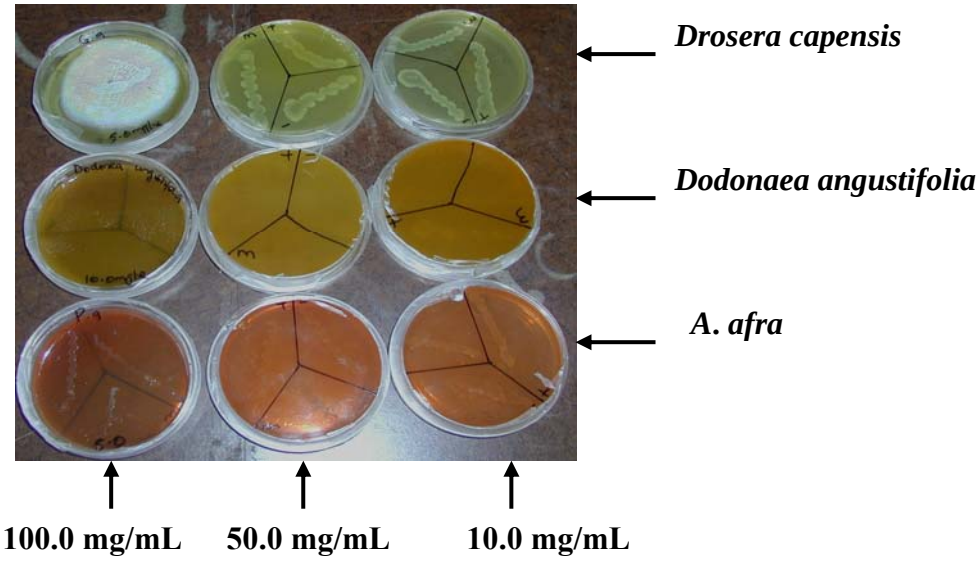


Figure 4.5. Antimycobacterial activity of *Drosera capensis*, *Dodonaea angustifolia* and *A. afra* against *M. smegmatis* using the agar plate method at 100.0 mg/mL

Table 4.1. Antimycobacterial activity of ethanol extracts against *M. smegmatis* using the agar plate method

Species	MIC[a] (mg/mL)
A. afra	100.0
Dodonaea angustifolia	100.0
Drosera capensis	100.0
G. africana	50.0
P. africana	na[b]
S. cordatum	na
Z. mucronata	na
Ciprofloxacin	0.01

[a]Minimum Inhibitory Concentration.

[b]Not active at highest concentration tested (200.0 mg/mL).

4.3.2. Minimum inhibitory concentration of selected plant extracts against *M. smegmatis* using the micro dilution method

Of the seven selected plants, the ethanol extract of *G. africana* was found to be the most effective against *M. smegmatis,* exhibiting a MIC of 0.78 mg/mL and a MBC of 1.56 mg/mL (Table 4.2). *A. afra* was the next best plant which inhibited growth at 1.56 mg/mL with a MBC of 6.25 mg/mL (Table 4.2). *Dodonaea angustifolia* and *Drosera capensis* had the same MIC of 3.12 mg/mL (Table 4.2; Figure 4.6). *S. cordatum* inhibited *M. smegmatis* at a concentration of 6.25 mg/mL. *P. africana* (Figure 4.6) and *Z. mucronata* were not active at the highest concentrations tested. The positive-control, ciprofloxacin, inhibited the growth of *M. smegmatis* at a MIC of 0.15 mg/mL and a MBC of 0.31 mg/mL (Table 4.2).

Table 4.2. Antimycobacterial activity of ethanol extracts against *M. smegmatis* using the micro dilution method

Species	MIC[a] (mg/mL)	MBC[b] (mg/mL)
A. afra	1.56	6.25
Dodonaea angustifolia	3.12	12.50
Drosera capensis	3.12	na
G. africana	0.78	1.56
P. africana	na[c]	nt[d]
S. cordatum	6.25	na
Z. mucronata	na	nt
Ciprofloxacin	0.15	0.31

[a]Minimum inhibitory concentration.

[b]Minimum bactericidal concentration.

[c]Not active at the highest concentration tested (6.25 mg/mL).

[d]Not tested for MBC determination.

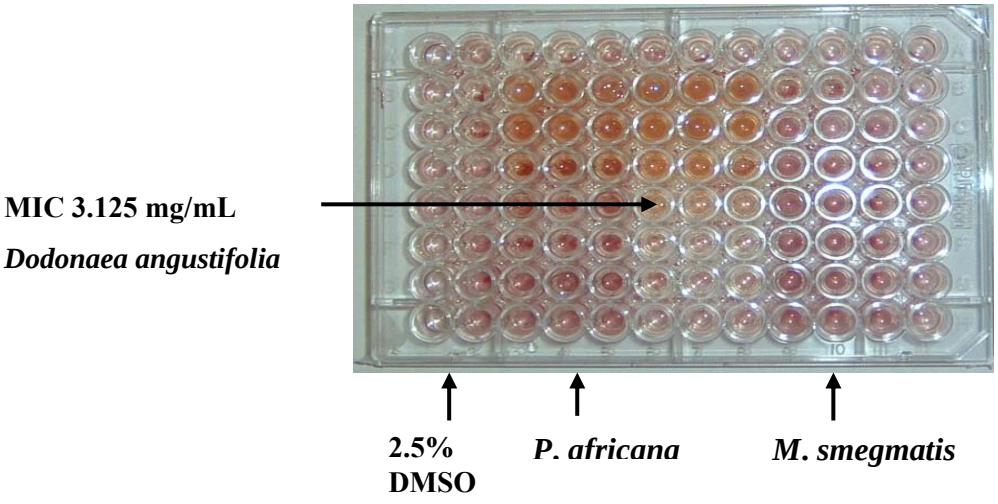


Figure 4.6. Microtitre plate exhibiting antimycobacterial activity against *M. smegmatis*

4.3.3. Minimum inhibitory concentration and bactericidal activity of selected medicinal plants against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method

The antimycobacterial assay of the ethanol extracts against *M. tuberculosis* using the BACTEC radiometric method showed that *G. africana* inhibited *M. tuberculosis* at a MIC of 1.2 mg/mL. In addition, *Dodonaea angustifolia* inhibited the bacteria at a concentration of 5.0 mg/mL whereas the other extracts did not show activity against *M. tuberculosis*. The antituberculosis positive drug, INH inhibited the growth of *M. tuberculosis* at 0.2 µg/mL (Table 4.3).

In the BACTEC system, the bactericidal effect of the various active extracts and the positive drug, were compared between the treated and untreated cultures. 100.0 µL of the bacterial suspensions from the BACTEC vials (exhibiting MICs), at the end of the experiment, was plated on 7H11 agar medium for a viable count enumeration. Only selected results (expressed as the mean viable counts ± standard error) in the case of treated and untreated vials are illustrated in figure 4.7. Both *Dodonaea angustifolia* and *G. africana* resulted in 1 log (90%) killing of the bacterial inoculum at a fixed concentration of 5.0 and 1.2 mg/mL respectively (Figure 4.7).

Table 4.3. Antimycobacterial activity of ethanol extracts of selected South African medicinal plants against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method

Species	MIC[a] (mg/mL)	ΔGI[b]
A. afra	na[c]	938.0 ± 86.2
Dodonaea angustifolia	5.0 (S[d])	0.5 ± 0.7
Drosera capensis	na[c]	200.5 ± 122.3
G. africana	1.2 (S)	0.0 ± 0.0
P. africana	na[c]	971.0 ± 39.5
S. cordatum	na[c]	919.0 ± 113.1
Z. mucronata	na[c]	839.0 ± 226.2
Isoniazid	2×10^{-4}	4.6 ± 2.8

[a]Minimum inhibitory concentration.

[b]ΔGI value (mean ± SD) of the control vial was 47.5 ± 9.0 for the sensitive strain.

[c]Not active at the highest concentration tested (5.0 mg/mL).

[d]Susceptible.

4.4. Discussion and Conclusion

One can conclude that of the different methods employed in this study, the microplate method gave the best indication of the potency of the selected plant species. There is a possibility that plant samples extracted with ethanol have more polar compounds as compared to non-polar compounds. Non polar compounds diffuse more slowly than polar compound in the aqueous agar medium and, thus, the results of the present study show weak activity. Another problem could be that, mycobacteria have a lipid-rich hydrophobic cell wall and are often susceptible to less polar compounds (Pauli *et al*., 2005). In solution, the extracts have more ability to have direct contact with the bacteria to work on them directly, while in an agar matrix there is not such a free interaction between the bacteria and the active extracts.

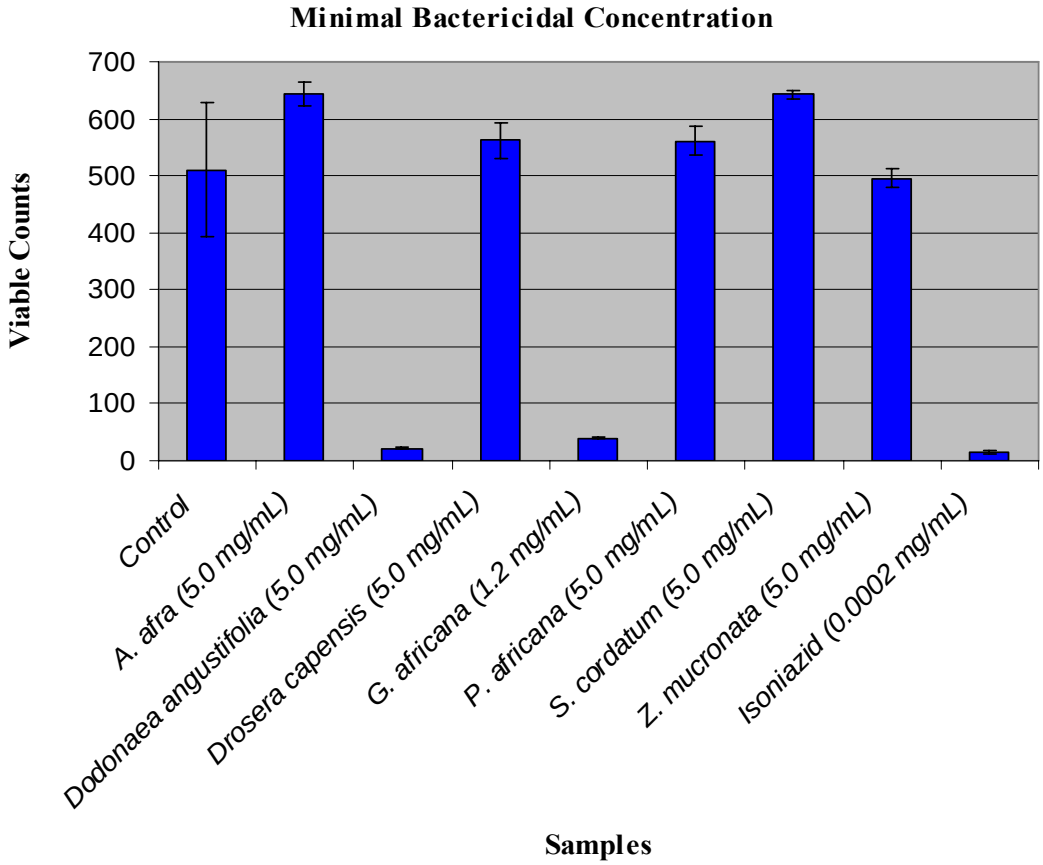


Figure 4.7. The comparative bactericidal effect of the active extracts against the drug-susceptibility strain of *M. tuberculosis*. Results illustrate the mean of viable bacterial counts ± standard error in the treated vials as compared to the untreated control vials

The ethanol extract of *G. africana* showed the best inhibitory effects when tested against *M. smegmatis* at 50.0 mg/mL and the positive control, ciprofloxacin, showed activity at 0.01 mg/mL concentration using the agar plate method. The ethanol extract of *A. afra* showed inhibition against *M. smegmatis* at 1.56 mg/mL but did not show activity against *M. tuberculosis* at the highest concentration (5.0 mg/mL) tested. In other studies, there were reports on the antimycobatcerial activity of other members of *Artemisia* at concentrations from 16.0 – 128.0 µg/mL (Fischer, 1996; Fischer *et al*., 1998). Graven *et al*., 1992, found no *in vitro* antimicrobial activity of aqueous extracts of South African collections of *A. afra* against *M. smegmatis* in disc assays. Asres *et*

al., 2001 investigating methanol extracts of *A*. *abyssinica* and *A*. *afra* tested against *M*. *tuberculosis* using the proportional method on Löwenstein-Jensen medium and found no activity at the highest concentration tested (4.0 mg/mL). Hexane and methanol extracts of *A*. *ludoviciana* were found to inhibit the growth of *M*. *tuberculosis* at a concentration of 200.0 µg/mL (Jimenez-Arellanes *et al*., 2003).

Other species of Asteraceae (*Arctotis auriculata*, *Eriocephalus africana*, *Felicia erigeroides* and *Helichrysum crispum*), were also evaluated for *in vitro* antimycobacterial activity against *M*. *smegmatis*, but only *A*. *auriculata* and *H*. *crispum* exhibited antimycobacterial properties at concentrations between 5.0 - 8.5 mg/mL (Salie *et al*., 1996). In the present study, *Dodonaea angustifolia* also did show antimycobacterial properties against both *Mycobacterium* species unlike the methanol extract tested by Asres *et al*., 2001 who found it to be inactive when concentrations were higher than 4.0 mg/mL. The antimycobacterial activity of *Drosera capensis* was observed only against *M*. *smegmatis* at a concentration of 3.12 mg/mL.

G. *africana* is widely used traditionally to treat tuberculosis, wounds and skin infections, and our findings show that this species has antimycobacterial activity. It is interesting to highlight that the other plants, *P*. *africana*, *S*. *cordatum* and *Z*. *mucronata*, reportedly used in traditional medicine to treat TB and other respiratory tract diseases, did not demonstrate any antimycobacterial activity against the two model organisms used in the present study. Similar results were obtained with species belonging to the family Rosaceae (*Prunus serotina* and *Rosa canina*) and Rhamnaceae (*Rhamnus cathartica*) (Newton *et al*., 2002). Eldeen and van Staden (2007), found no activity of ethanol extract of *P*. *africana* when tested against another mycobacterium species (*M*. *aurum*).

It may be that these plants are used to treat the symptoms of TB rather than actually for curing the disease itself. Some plant species may not contain compounds that inhibit the growth of *M*. *smegmatis* or *M*. *tuberculosis* but it is possible that they may have stimulant or modulatory effects on the immune system. In addition, factors including climate, soil type, and the season in which the plants were collected and the

storage conditions may also affect the chemical composition of plants. Activities of the plant extracts *in vitro* may not be parallel to those *in vivo*, and this gives rise to the possibility that potentially useful compounds may be missed (Newton *et al*., 2002).

In conclusion, our findings indicated some correlation between the activities of the ethanol plant extracts when screened against both *M. smegmatis* and *M. tuberculosis*. Selection of plants by ethnobotanical criteria offers a good probability of finding candidates which contain compounds active against mycobacteria (Cantrell *et al*., 1998; Lall and Meyer, 1999; McCutcheon *et al*., 1997; Van Puyvelde *et al*., 1994). Based on the present study, it can be concluded that the ethanol extract of *G. africana* can be considered as a candidate for further investigation in pre-clinical trials, for its potential as a antimycobacterial agent.

CHAPTER 5
CYTOTOXICITY ACTIVITY OF SELECTED SOUTH AFRICAN MEDICINAL PLANTS AGAINST VERO CELLS

5.1. Introduction

Epidemiological statistics show that cancer and infectious diseases like tuberculosis (TB) are important causes of mortality throughout the world (WHO, 1999). Secondary metabolites of plants possess many biological activities since they serve either as protective agents against various pathogens (e.g. insects, fungi and bacteria) or as growth regulatory molecules (e.g. hormone-like substances that stimulate or inhibit cell division and morphogens). These physiological effects make some of them potentially anti-cancerous, due to either their direct cytotoxicity on cancer cells or modulation of tumor development (Don *et al*., 2006). Cytotoxicity tests are part of developing a potential pharmaceutical product into a clinically acceptable drug. This provides a screening system to ascertain that the compounds being tested are not more harmful to the normal biological processes than the effects they are being tested for (Gebhardt, 2000). Various natural products have particular reactions against biological systems and the cytotoxic evaluation of plant extracts is essential before they could be considered for new drug development (Avila *et al*., 1997). Many plant extracts and isolated compounds have been tested *in vitro* for cytotoxicity by using different human cell lines (prostate, stomach, cancer, liver colon, etc) as well as animal cells such as monkey kidney cells (Lamidi *et al*., 2005; Al-Fatimi *et al*., 2005).

In this chapter, the cytotoxicity activity of the crude extracts from the selected medicinal plants against Vero cell lines is reported.

5.2. Materials and methods

5.2.1. Plant materials

Plant species and their parts were selected based on their traditional use for TB. The selection of plant species for this study was based on the information culled from published sources and traditional healers. (Chapter 3, Table 3.1; Chapter 4, section 4.2.1). Voucher specimens were deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa (Chapter 3, Table 3.1).

5.2.2. Preparation of plant extracts

Preparations of crude extracts were prepared according to Chapter 4, section 4.2.2.

5.3. Cells culture

5.3.1. Vero cell line

The cytotoxicity of all the crude ethanol extracts against Vero cells were tested following the method of Zheng *et al*., 2001. Cells were cultured in Eagle's minimal essential (MEM**),** supplemented with 1.5 g/L sodium bicarbonate, 2mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/mL penicillium, 10.0 µg/mL streptomycin, 0.25 µg/mL fungizone and 10% fetal bovine serum, at 37^{o}C in a humidified atmosphere with 5% CO_2. Cells were subcultured in a ratio of 1:6 every second to third day after the trypsinization of confluent cultures (Zheng *et al*., 2001).

5.4. Cytotoxicity assay

Cytotoxicity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-

[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide (XTT) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). 100.0 µL of Vero cell lines ($1x10^5$ Cells/mL) were seeded into inner wells of the microtiter plates, while in the outer wells 200.0 µL of incomplete medium was added. The plates were incubated for 24 hours to allow the cells to attach to the bottom of the plate.

Dilution series were made of the extracts and the various concentrations (400.0 to 3.12 µg/mL) were added to the inner wells of the microtiter plate and incubated for 48 hours (Figure 5.1). Fifty microlitters of XTT reagent (1.0 mg/mL XTT with 0.383 mg/mL PBS) was added to the wells and the plates were then incubated for 1 - 2 hours. The positive control, (Zearalenone) was included at final concentration of 1.25 µg/mL. After incubation the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader, which measures the optical density at 450 nm with a reference wavelength of 690 nm. Referring to the control (medium with DMSO control), cell survival was assessed. The 'GraphPad Prism 4', statistical program was used to analyse the fifty percent inhibitory concentration (IC_{50}) values.

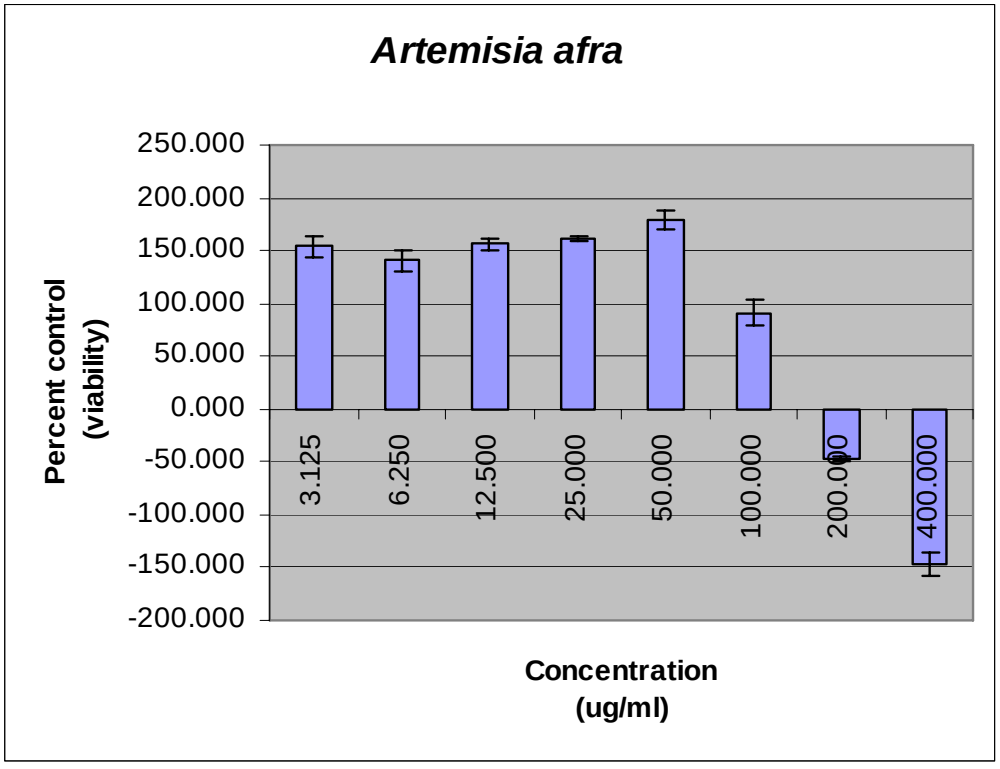
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		**A**								Media	DMSO	
C		400	200	100	50	25	12.5	6.25	3.125	Media	DMSO	
D		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	Media	DMSO	
E		**B**								Media	DMSO	
F		400	200	100	50	25	12.5	6.25	3.125	Media	DMSO	
G		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	Media	DMSO	
H												

Figure 5.1. Microtitre plate for cytotoxicity testing

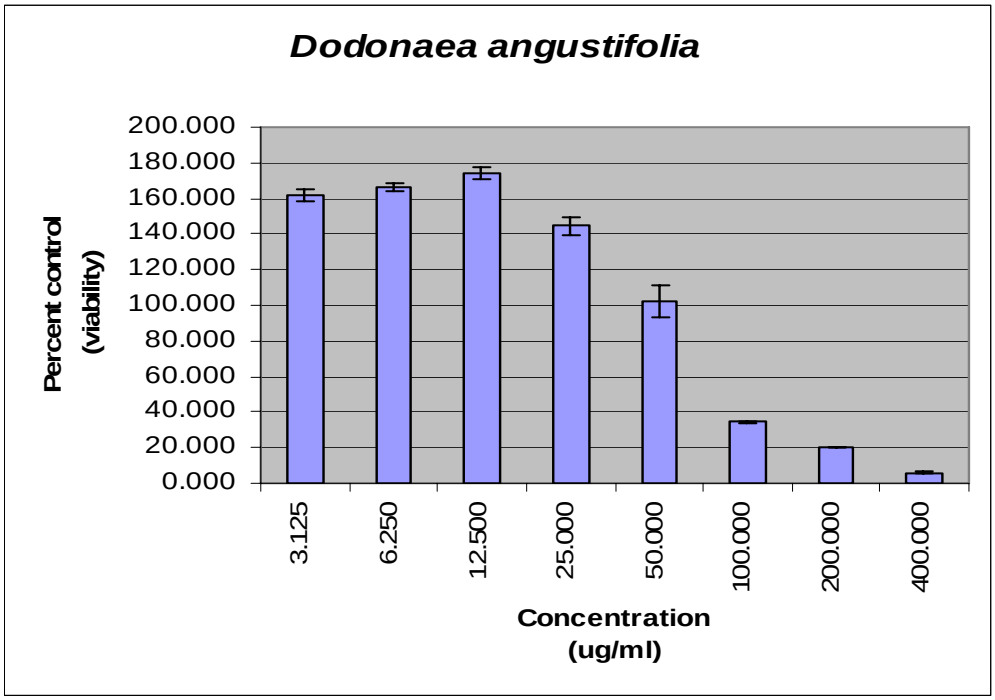
5.5. Results and Discussion

A cytotoxicity assay is a rapid and cost-effective tool to sort out the likely failures before a plant extract or a compound enters into the costly development process and also help to choose the optimal candidate. The results (Figure 5.2) obtained indicated that ethanol crude extracts of the selected plants have moderate toxicity. The cytotoxicity effects of the six ethanol plant extracts on Vero cells demonstrated marginal toxicity except for *S. cordatum*, which showed fifty percent inhibitory concentration (IC_{50}) at 2.67 µg/mL against the Vero cells. Ethanol extract of *P. africana* had the highest IC_{50} value (212.0 µg/mL) compared to other plant extracts. *G. africana* showed moderate toxicity exhibiting IC_{50} at 101.3 µg/mL (Table 5.1).

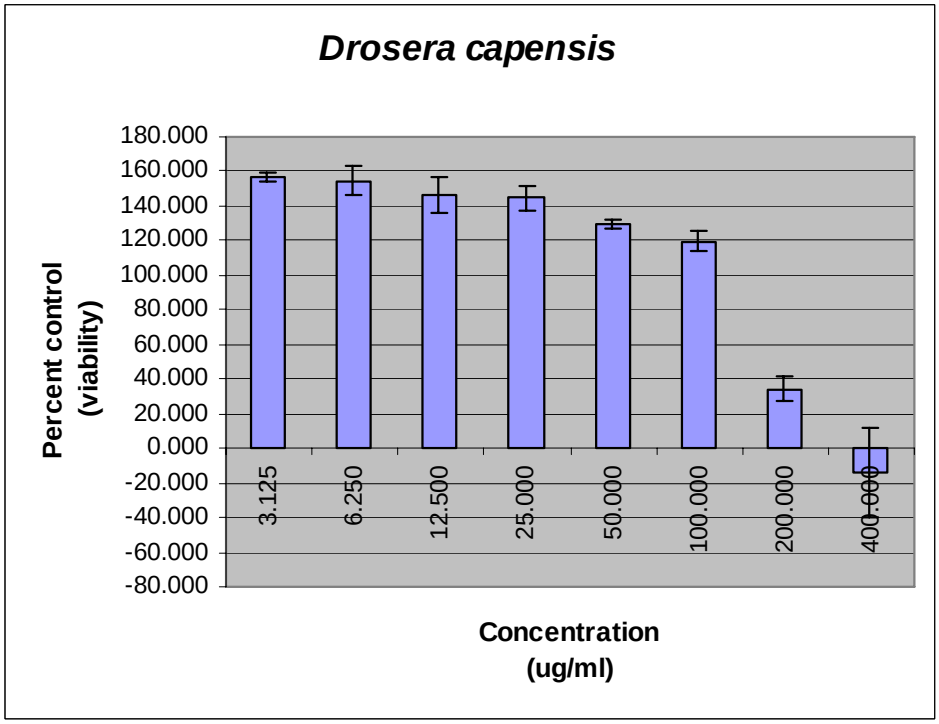
The dose-response curves of each plant extract against the proliferation of Vero cells are as follows:



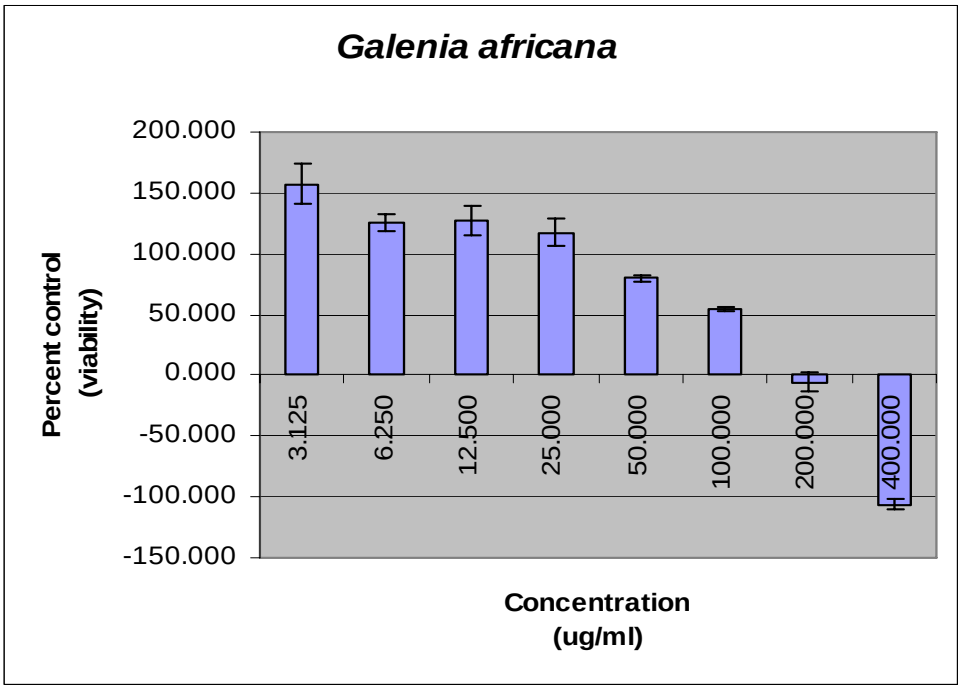
(a)


Dodonaea angustifolia
Percent control (viability)
200.000
180.000
160.000
140.000
120.000
100.000
80.000
60.000
40.000
20.000
0.000
3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000
Concentration (ug/ml)

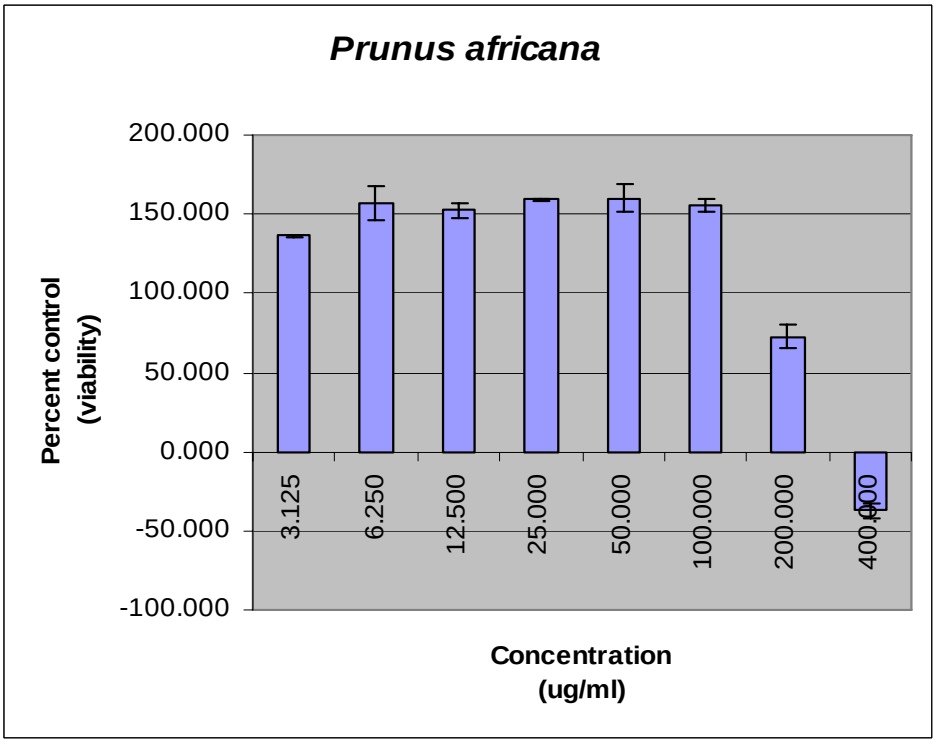
(b)


Drosera capensis
Percent control (viability)
180.000
160.000
140.000
120.000
100.000
80.000
60.000
40.000
20.000
0.000
-20.000
-40.000
-60.000
-80.000
3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000
Concentration (ug/ml)

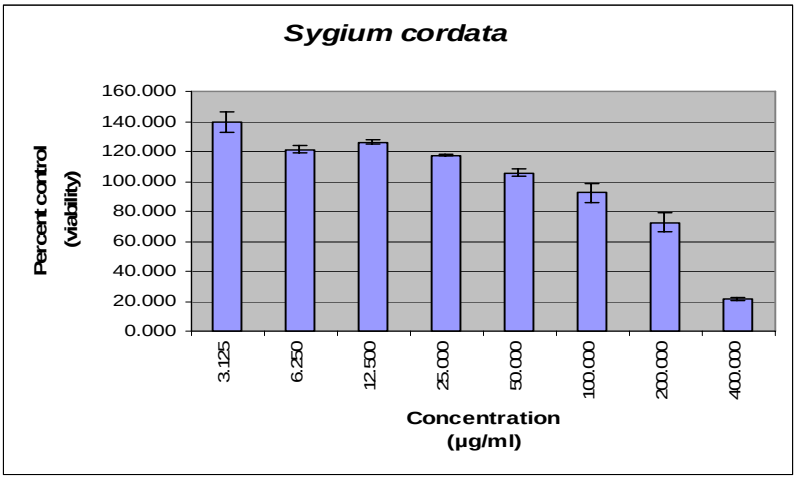
(c)


Galenia africana
200.000
150.000
100.000
50.000
0.000
-50.000
-100.000
-150.000
Percent control (viability)
3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000
Concentration (ug/ml)

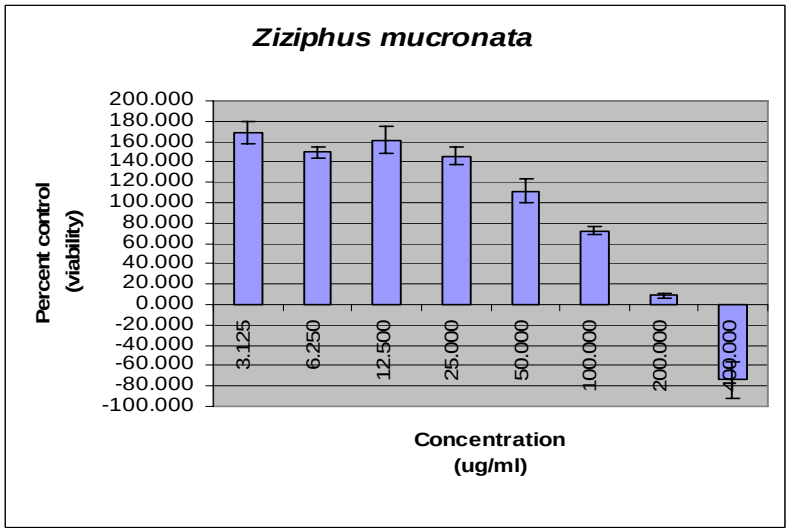
(d)


Prunus africana
200.000
150.000
100.000
50.000
0.000
-50.000
-100.000
Percent control (viability)
3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000
Concentration (ug/ml)

(e)



(f)



(g)

Figure 5.2. Effect of the ethanol crude extracts of selected South African medicinal plants on the proliferation of Vero cells

(a) ***A. afra***

(b) ***D. angustifolia***

(c) ***D. capensis***

(d) ***G. africana***

(e) ***P. africana***

(f) ***S. cordata***

(g) ***Z. mucronata***

Table 5.1. Cytotoxicity of the ethanol extracts of selected South African medicinal plants against Vero cells

Plant species	IC_{50}[a] (µg/mL ± SD)	MIC[b] (µg/mL)	SI[c]
A. afra	113.0 ± 2.05	na[d]	nd[e]
Dodonaea angustifolia	091.0 ± 1.10	5×10^3	0.018
Drosera capensis	141.4 ± 2.14	na	nd
G. africana	101.3 ± 2.19	1.2×10^3	0.084
P. africana	212.0 ± 2.33	na	nd
S. cordatum	002.67 ± 2.54	na	nd
Z. mucronata	118.2 ± 2.36	na	nd
Zearalenone (positive control)	002.31 ± 0.30	nd	nd

[a]IC_{50}, 50% inhibitory concentration of samples on Vero cell line.

[b]Minimum inhibitory concentration against *M. tuberculosis*.

[c]SI, selectivity index (*in vitro*): IC_{50} in Vero cells/MIC against *M. tuberculosis*.

[d]Not active at the highest concentration tested (5.0 mg/mL) against *M. tuberculosis*.

[e]Not determined.

5.6. Conclusion

Cytotoxicity screening of plant-derived extracts is a necessary aspect of the preliminary safety evaluation for further testing or compound isolation. This helps to ensure that the biological activity of the plant extract is not due to a general toxic effect. The *in vitro* cytotoxicity in early research efforts provides an important advantage in identifying potentially cytotoxic compounds. However, *in vitro* cytotoxic assays that can reliably predict *in vivo* toxicity of a drug is rare because it is the most difficult property to adequately investigate. Nonetheless, these assays do provide a useful tool to compare and rank new non-poisonous compounds to some extent (Hamid *et al*., 2004).

Dodonaea angustifolia and *G. africana* displayed activity against *M. tuberculosis* but they also displayed lower IC_{50} values in Vero cells. *A. afra* was evaluated against for anticancer activity against human cell lines (breast MCF7, renal TK10 and melanoma UACC62) and was found to have total inhibition growth of 9.73 µg/mL for UACC62 (Fouche *et al*., 2008). This is the first report on cytotoxicity of plant samples selected in this study on Vero cell lines.

CHAPTER 6

BIOASSAY GUIDED FRACTIONATION OF *G. AFRICANA* L. VAR. *AFRICANA*

6.1. Introduction

There is no information on isolated constituents from plants belonging to the genus Galenia. Preliminary chemical tests of *G. africana* showed presence of alkaloids but not of saponnins, tannins and glycosides. Compounds such as dihydroechinoidinin, 2', 4'dihydroxydihydrochalcone, 5,7-dihydroxy-flavanone (pinocembrin), 2',4'-dihydroxychalcone and 5-hydroxy-7-methoxy-flavanone (pinostrobin) have been isolated from *G. africana* (Figure 6.1). These compounds were investigated for antifungal properties (Vries *et al*.,2005). However, there is no current information regarding other secondary metabolites from *G. africana*.

G. africana has been associated with liver damage and severe ascites, a condition commonly referred to as "waterpens" in sheep and goats. Indigenous tribes chew the plant to relieve toothache and it has been reported that it can produce blisters on the mucous membrane of the mouth if used excessively. The plant is also used in the treatment of venereal diseases and a decoction as a lotion for skin diseases, including ringworm, and for the relief of inflammation of the eyes (Adamson, 1956). The objective in this chapter is to isolate, purify and characterize the secondary metabolites with antimycobacterial activity from the leaves of *G. africana*.

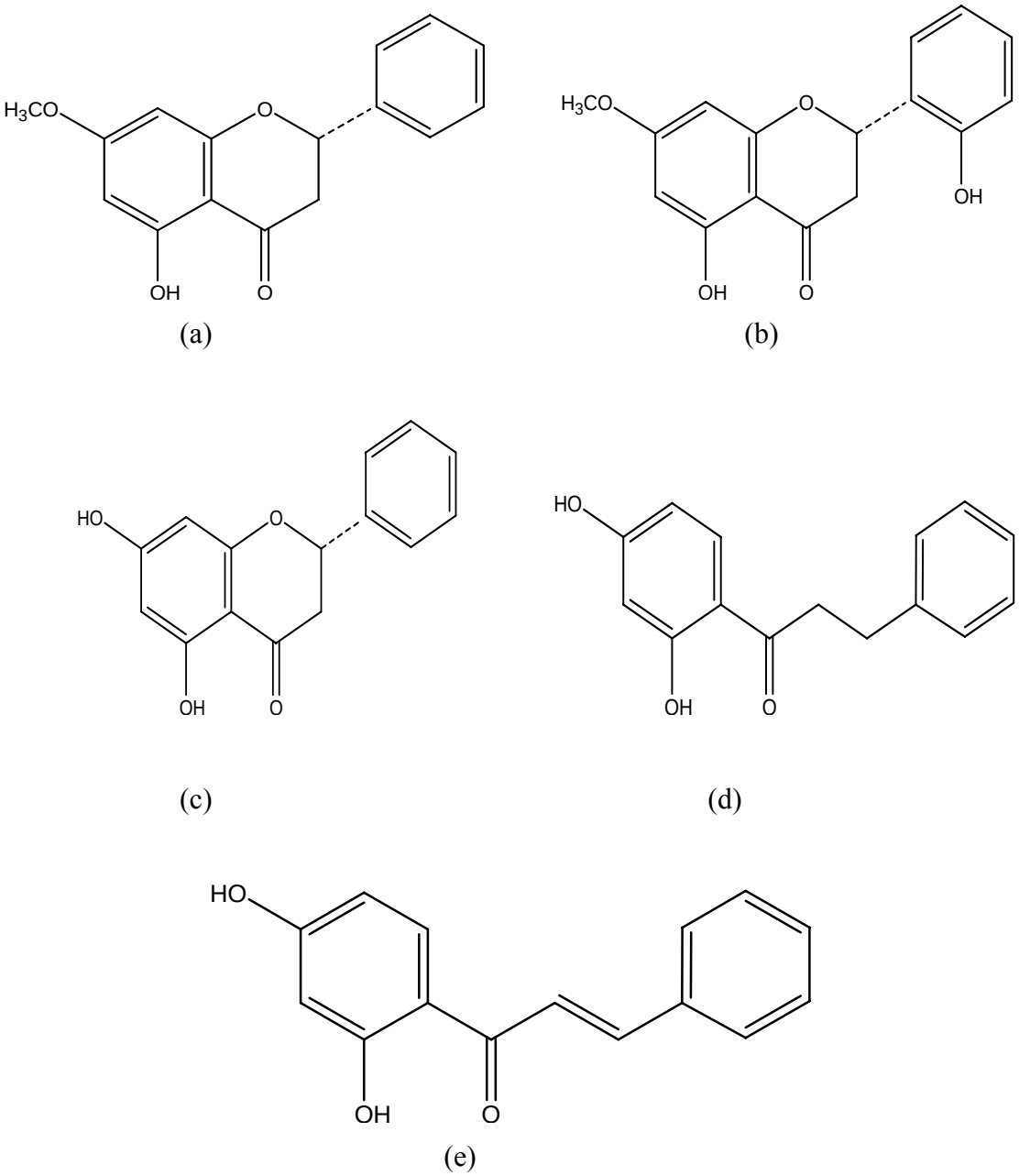


Figure 6.1. Chemical structure of compounds isolated from *G. africana*

(a) 5-hydroxy-7-methoxy-flavanone (pinostrobin)

(b) Dihydroechinoidinin

(c) 5,7-dihydroxy-flavanone(pinocembrin)

(d) 2',4'dihydroxydihydrochalcone

(e) 2',4'-dihydroxychalcone

6.2. Bioassay guided fractionation

6.2.1. Silica gel column and GPC-HPLC chromatography

Considering significant inhibitory activity shown by the ethanol extract of *G. africana* against *M. smegmatis* and *M. tuberculosis*, (Chapter 4), this plant species was selected for the identification of bioactive principles. The ethanol crude extract was filtered and evaporated under reduced pressure. The total concentrated extract (20.0 g) which appeared as a yellow-green solid, was soluble in ethanol (EtOH), methanol (MeOH), and dichloromethane (CH_2Cl_2), but insoluble in hexane ($CH_3(CH_2)_4CH_3$) and partially soluble in water (H_2O). A portion of the ethanol extract (6.0 g) was chromatographed on silica gel column chromatography PF_{254} (CC, size 70 x 120 cm, 2.5 kg, Figure 6.2) using $CH_3(CH_2)_4CH_3$/ethyl acetate (EtOAc) mixtures of increasing polarity (0 to 100%) followed by 100% EtOH. In total, 21 crude fractions (300.0 mL) were collected (Figure 6.3) and analysed on thin layer chromatographic (TLC) using different mixtures of $CH_3(CH_2)_4CH_3$: EtOAc. Similar fractions, according to the (TLC) profile, were combined which resulted into 6 main fractions (Figure 6.4). The fractions were assayed on a thin layer chromatogram (TLC silica gel 60 F_{254}) using $CH_3(CH_2)_4CH_3$ and EtOAc (8:2) as eluent and analysed under a UV light. The rest of the compounds remaining on the column were washed out with 2.0 L of 100% EtOAc. All 6 main fractions were tested against *M. smegmatis* and *M. tuberculosis* (Chapter 7).

The GPC-HPLC (gel permeation chromatography-high pressure liquid chromatography, 20.0 mm x 500.0 mm, flow rate of 1.0 mL/min, temperature of 40^{o}C and wavelength of 206nm) isolation was carried out by LC-908W (Japan Analytical Industry). GPC-HPLC is a normal HPLC method by the use of GPC column instead of octadecyl silane (ODS) or silica gel column. NMR spectra were recorded on JNM ECA-600 (JEOL). Chemical shifts for ^{1}H and ^{13}C NMR are given in parts per million (δ) relative to tetramethylsilane (δ_H) and residual solvent signals (δ_C 49.0 and 29.8 for methanol-d_4 and acetone-d_6, respectively) as internal standards. Mass spectra were

measured on JMS AX-500 and AX-700 JEOL (Figure 6.6).

Schematic representation of the purification steps for the isolation of the compounds is illustrated in Figure 6.5.

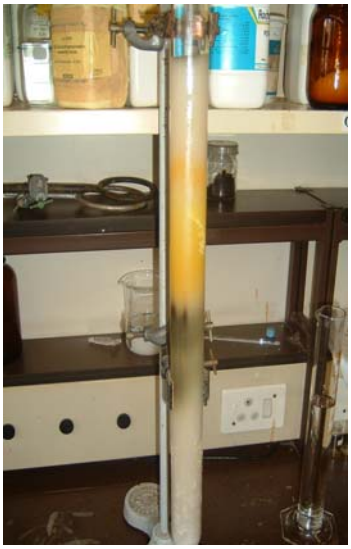
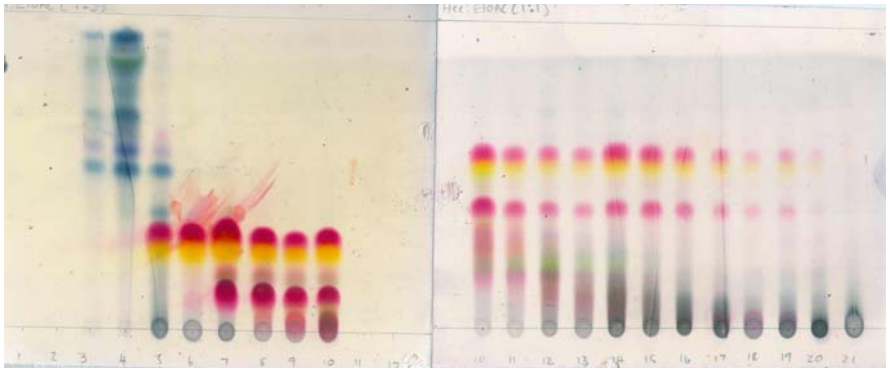


Figure 6.2. Chromatographic purification of the ethanol extract of *G. africana* using silica column chromatography PF_{254}



(a) **(b)**

Figure 6.3. TLC developed-plates of 21 main fractions obtained from silica column. Solvent systems: (a) $CH_3(CH_2)_4CH_3$: EtOAc (3:1)
(b) $CH_3(CH_2)_4CH_3$: EtOAc (3:2)
Detection:Vanillin in H_2SO_4

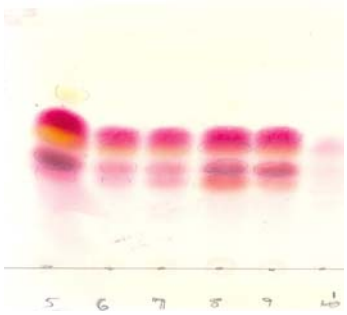


Figure 6.4. **TLC developed-plates of similar and combined fractions which resulted in 6 main fractions. Solvent systems: $CH_3(CH_2)_4CH_3$: EtOAc (8:2). Detection : Vanillin in H_2SO_4**

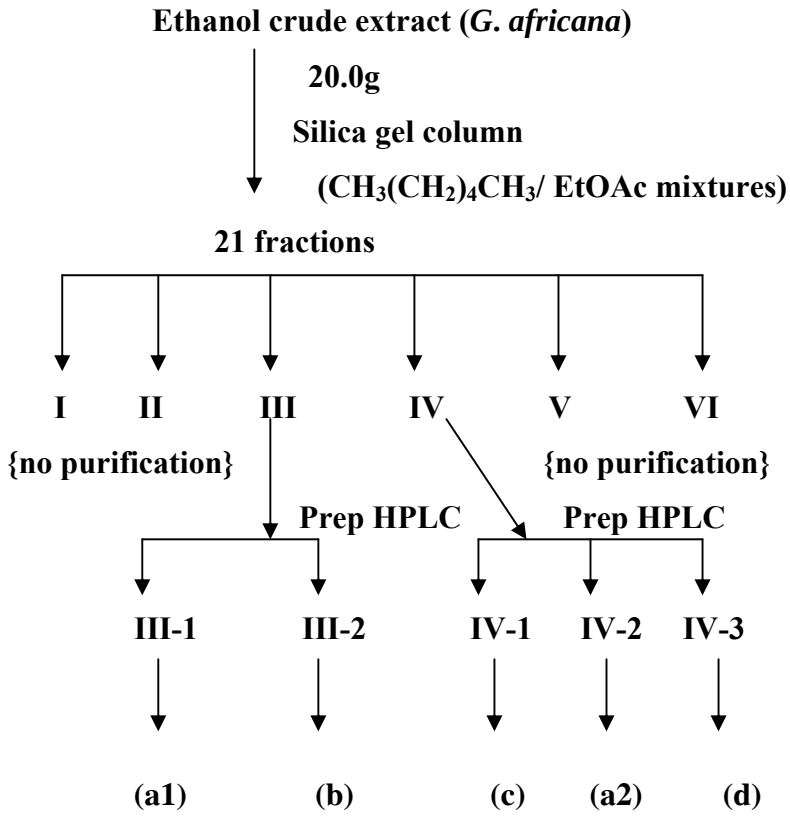


Figure 6.5. **Schematic representation of the purification steps for the isolation of the compounds from the ethanol extract of *G. africana***

(a1): (2*S*)-5,7,2'-trihydroxyflavanone

(b): (*E*)-3,2',4'-trihydroxychalcone

(c): (*E*)-2',4'-dihydroxychalcone

(a2): (2*S*)-5,7,2'-trihydroxyflavanone

(d): (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

6.3. Results and Discussion

Fractions I (81.0 mg) and II (56.0 mg) did not show presence of compounds whereas fractions III (112.9 mg), IV (201.2 mg) and V (0.28 mg) which had similar TLC profiles showed presence of different compounds. Fractions III and IV were subjected to further purification and were individually chromatographed on GPC-HPLC, eluting with $CHCl_3$-MeOH (4:1). The major fractions of fractions III and IV were shown by TLC and NMR to be mixtures containing varying proportions of chalcone, dihydrochalcone and flavanone. Fraction V was not sufficient and therefore it was not selected for purification. Three known and one novel compounds belonging to flavonoid and chalcone groups were isolated from fractions III and IV of the ethanol extract of *G. africana*. The structural analysis of the isolated compounds is as follows:

Fraction III (112.9 mg) was subjected to GPC-HPLC (column, JAIGEL-GS310 (f 20.0 mm x 500 mm); solvent, $CHCl_3$-MeOH (4:1); flow rate, 5.0 mL/min.) to give 2 fractions: Fraction III-1 (108.0 mg, t_R 13.6~23.4 min.), was a pure compound, **(2*S*)-5,7,2'-trihydroxyflavanone** (Figure 6.7a). Fraction III-2 (33.7 mg, t_R 25.1~29.0 min.), was subjected to GPC-HPLC (column, JAIGEL-W252 (f 20.0 mm x 500 mm); solvent, $CHCl_3$-MeOH (4:1); flow rate, 5 mL/min.) to give **(*E*)-3,2',4'-trihydroxychalcone** (Figure 6.7c).

Fraction IV (201.2 mg) was subjected to GPC-HPLC (column, JAIGEL-GS310 (f 20.0 mm x 500 mm); solvent, $CHCl_3$-MeOH (4:1); flow rate, 5.0 mL/min.) to give 3 fractions: Fraction IV-1 (11.0 mg, t_R 20.3~21.7 min.), was subjected to GPC- HPLC (column, JAIGEL-W252 (f 20.0 mm x 500 mm); solvent, $CHCl_3$-MeOH (4:1); flow rate, 5.0 mL/min.) to give **(*E*)-2',4'-dihydroxychalcone** (4.0 mg, t_R 12.2~16.5 min.) (Figure 6.7b). Fraction IV-2 (80.5 mg, t_R 24.0~26.9 min.), was a pure compound, **(2*S*)-5,7,2'-trihydroxyflavanone** (30.0 mg). Fraction IV-3 (28.3 mg, t_R 26.9~29.4 min.), was subjected to GPC-HPLC (column, JAIGEL-W252 (f 20.0 mm x 500 mm); solvent, $CHCl_3$-MeOH (4:1); flow rate, 5.0 mL/min.), and the peaks between tR 11.7~15.1 min. were separated by the recycle mode. After five times of cycles,

(*E*)-3,2',4'-trihydroxy-3'-methoxychalcone (18.0 mg) was isolated (Figure 6.7d). The aromatic signals and structure elucidation of each purified compound are as follows:

(2*S*)-5,7,2'-trihydroxyflavanone: colourless amorphous solid; $[\alpha]_D$ -114 (*c* 1.00, MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ 7.44 (1H, dd, *J* = 7.7, 1.4 Hz), 7.14 (1H, td, *J* = 7.7, 1.4 Hz), 6.86 (1H, td, *J* = 7.7, 0.8 Hz), 6.81 (1H, dd, *J* = 7.7, 0.8 Hz), 5.94 (1H, d, *J* = 2.2 Hz), 5.89 (1H, d, *J* = 2.2 Hz), 5.68 (1H, dd, *J* = 12.8, 3.1 Hz), 2.94 (1H, dd, *J* = 17.2, 12.8 Hz), 2.80 (1H, dd, *J* = 17.2, 3.1 Hz) (Figure 6.5b). ^{13}C NMR (150 MHz, CD_3OD) δ 198.0, 168.2, 165.4, 165.1, 155.2, 130.2, 127.6, 126.7, 120.7, 116.2, 103.3, 97.1, 96.2, 75.9, 42.9; EIMS *m/z* 272 $[M]^+$, 254 (100%), 253, 179, 153; HREIMS *m/z* 272.0667 (272.0685 calcd for $C_{15}H_{12}O_5$); CD (MeOH) λ, nm ([θ]): 286 (-43800), 315 (+5210), 325 (+6840) (Figure 6.5a).

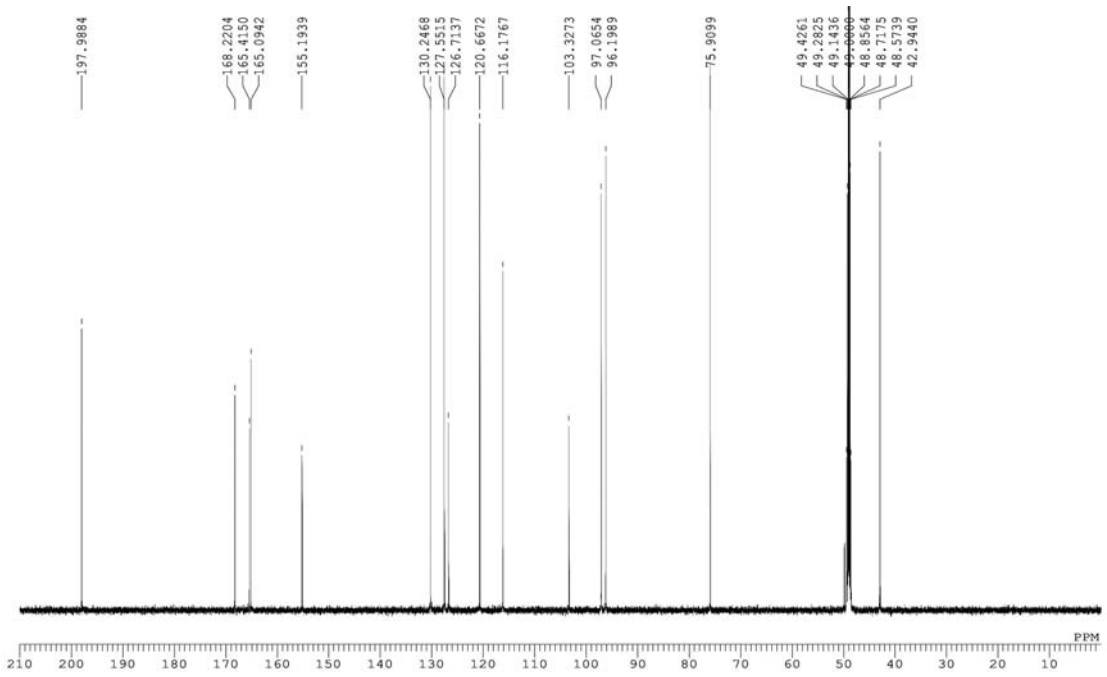
(*E*)-2',4'-dihydroxychalcone: yellow amorphous solid; the spectrum was of a chalcone structure, with ^{1}H NMR (600 MHz, acetone-d_6) δ 13.45 (1H, s), 8.15 (1H, d, *J* = 8.9 Hz), 7.95 (1H, d, *J* = 15.4 Hz), 7.87 (1H, d, *J* = 15.4 Hz), 7.84-7.87 (2H, m), 7.44-7.48 (3H, m), 6.48 (1H, dd, *J* = 8.9, 2.4 Hz), 6.37 (1H, d, *J* = 2.4 Hz) (Figure 6.5d). ^{13}C NMR (150 MHz, acetone-d_6) δ 192.9, 167.8, 166.1, 144.9, 136.1, 133.7, 131.5, 129.9 (2C), 129.8 (2C), 121.9, 114.6, 109.1, 103.9; EIMS *m/z* 240 $[M]^+$ (100%), 223, 163, 137; HREIMS *m/z* 240.0783 (240.0786 calcd for $C_{15}H_{12}O_3$) (Figure 6.5c).

(*E*)-3,2',4'-trihydroxychalcone (not reported from natural sources): yellow amorphous solid; ^{1}H NMR (600 MHz, acetone-d_6) δ 13.45 (1H, s), 8.14 (1H, d, *J* = 8.9 Hz), 7.87 (1H, d, *J* = 15.5 Hz), 7.79 (1H, d, *J* = 15.5 Hz), 7.32 (1H, dt, *J* = 7.7, 1.1 Hz), 7.26-7.30 (2H, m), 6.94 (1H, ddd, *J* = 8.0, 2.4, 1.1 Hz), 6.48 (1H, dd, *J* = 8.9, 2.5 Hz), 6.37 (1H, d, *J* = 2.5 Hz) (Figure 6.5f). ^{13}C NMR (150 MHz, acetone-d_6) δ 192.8, 167.7, 165.9, 158.8, 144.9, 137.7, 133.6, 130.8, 121.7, 121.1, 118.6, 116.1, 114.4, 108.9, 103.8; EIMS *m/z* 256 $[M]^+$ (100%), 239, 163, 137; HREIMS *m/z* 256.0722 (272.0736 calcd for $C_{15}H_{12}O_4$) (Figure 6.5e).

(*E*)-3,2',4'-Trihydroxy-3'-methoxychalcone (Novel compound): yellow amorphous solid; ^{1}H NMR (600 MHz, acetone-d_6) δ 13.67 (1H, s, 2-OH), 7.94 (1H, d, J = 9.1 Hz, H-6'), 7.87 (1H, d, J = 15.4 Hz, H-α), 7.80 (1H, d, J = 15.4 Hz, H-β), 7.33 (1H, dt, J = 7.7, 1.0 Hz, H-4), 7.26-7.30 (2H, m, H-2,5), 6.94 (1H, ddd, J = 8.0, 2.5, 1.0 Hz, H-6), 6.52 (1H, d, J = 9.1 Hz, H-5'), 3.85 (3H, s, 3'-OMe) (Figure 6.3h). ^{13}C NMR (150 MHz, acetone-d_6) δ 193.5 (C=O), 159.8 (C-2'), 158.8 (C-3), 157.9 (C-4'), 145.1 (C-β), 137.2 (C-1), 135.8 (C-3'), 130.8 (C-5), 127.7 (C-6'), 121.6 (C-α), 121.2 (C-4), 118.7 (C-6), 116.1 (C-2), 115.2 (C-1'), 108.4 (C-5'), 60.5 (3'-OMe); EIMS *m/z* 286 $[M]^+$ (100%), 225, 193, 166, 138; HREIMS *m/z* 286.0829 (272.0841 calcd for $C_{16}H_{14}O_5$) (Figure 6.5g).

HREIMS (*m/z* 286.0829 $[M^+]$) indicated the molecular formula of (*E*)-3,2',4'-Trihydroxy-3'-methoxychalcone as $C_{16}H_{14}O_5$. ^{1}H, ^{13}C NMR and HMQC spectra indicated that (*E*)-3,2',4'-Trihydroxy-3'-methoxychalcone was the kind of chalcones including a 1,3-disubstituted benzene ring (δ_H 7.33 (1H, dt, J = 7.7, 1.0 Hz), δ_H 7.26-7.30 (2H, m) and δ_H 6.94 (1H, ddd, J = 8.0, 2.5, 1.0 Hz)), a 1,2,3,4-tetrasubstituted benzene ring (δ_H 7.94 (1H, d, J = 9.1 Hz) and δ_H 6.52 (1H, d, J = 9.1 Hz), a methoxyl group (δ_C 60.5 and δ_H 3.85) and an α,β-unsaturated ketone (δ_C 193.5; δ_C 121.6 and δ_H 7.87 (1H, d, J = 15.4 Hz); δ_C 145.1 and δ_H 7.80 (1H, d, J = 15.4 Hz)). HMBC spectrum revealed the connection of these constituents to elucidate the structure of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone (Figure 6.8)

Compounds, **(2*S*)-5,7,2'-trihydroxyflavanone** and **(*E*)-2',4'-dihydroxychalcone** were reported previously (Miyachi *et al*., 1987, Su *et al*., 2003, Miyaichi and Morimoto, 2006, Miyachi *et al*., 2006, Vries *et al*., 2005, Zampini *et al*., 2005 and Svetaz *et al*., 2007). **(*E*)-3,2',4'-trihydroxychalcone** was synthesized previously (Severi *et al*., 1998), but has not been reported from natural sources.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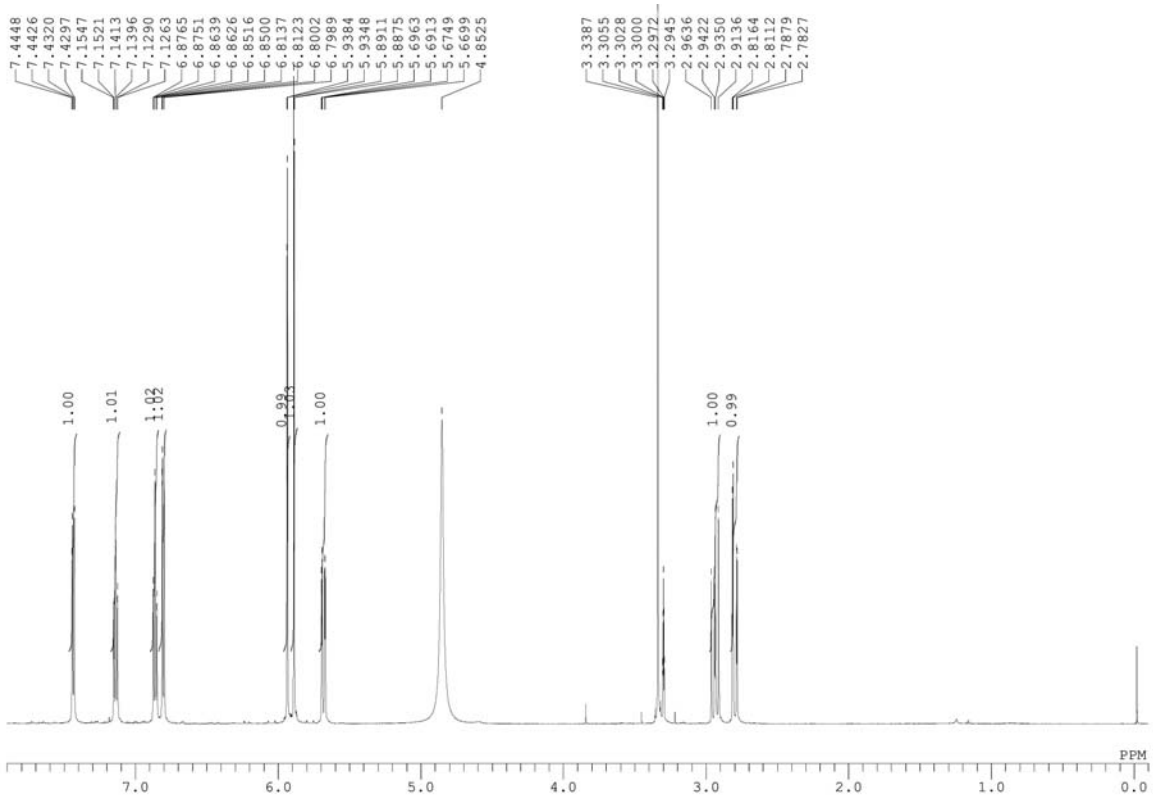
(a)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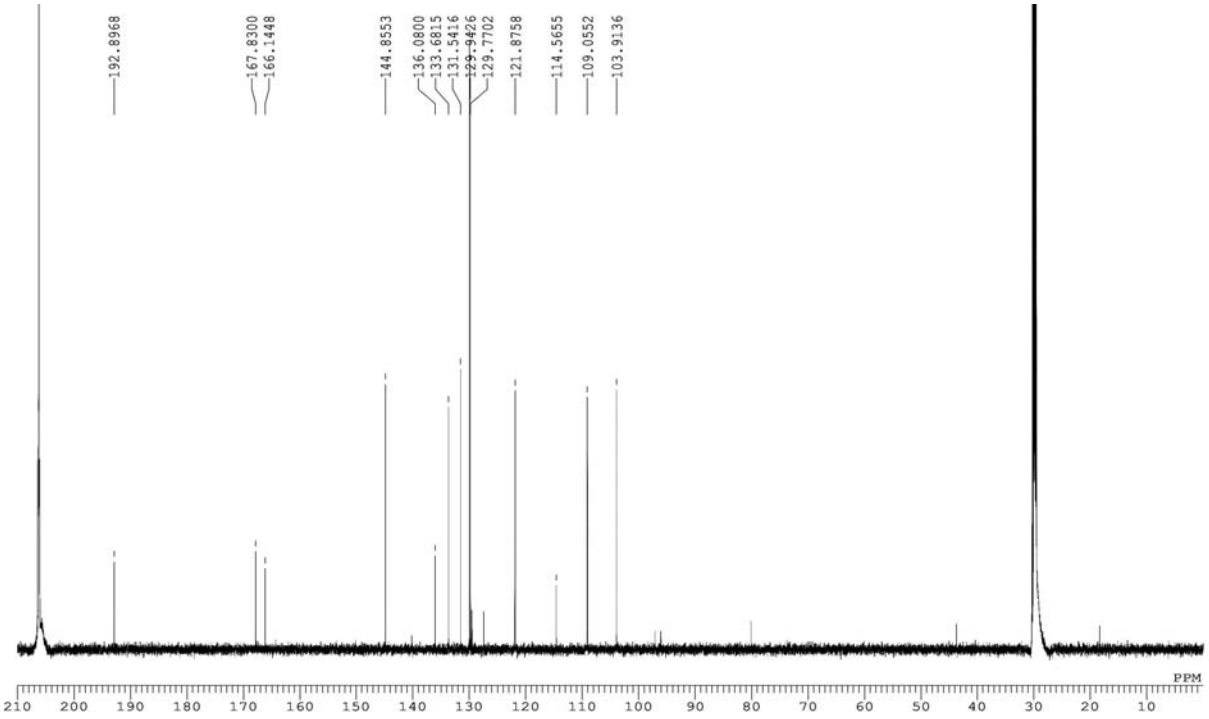
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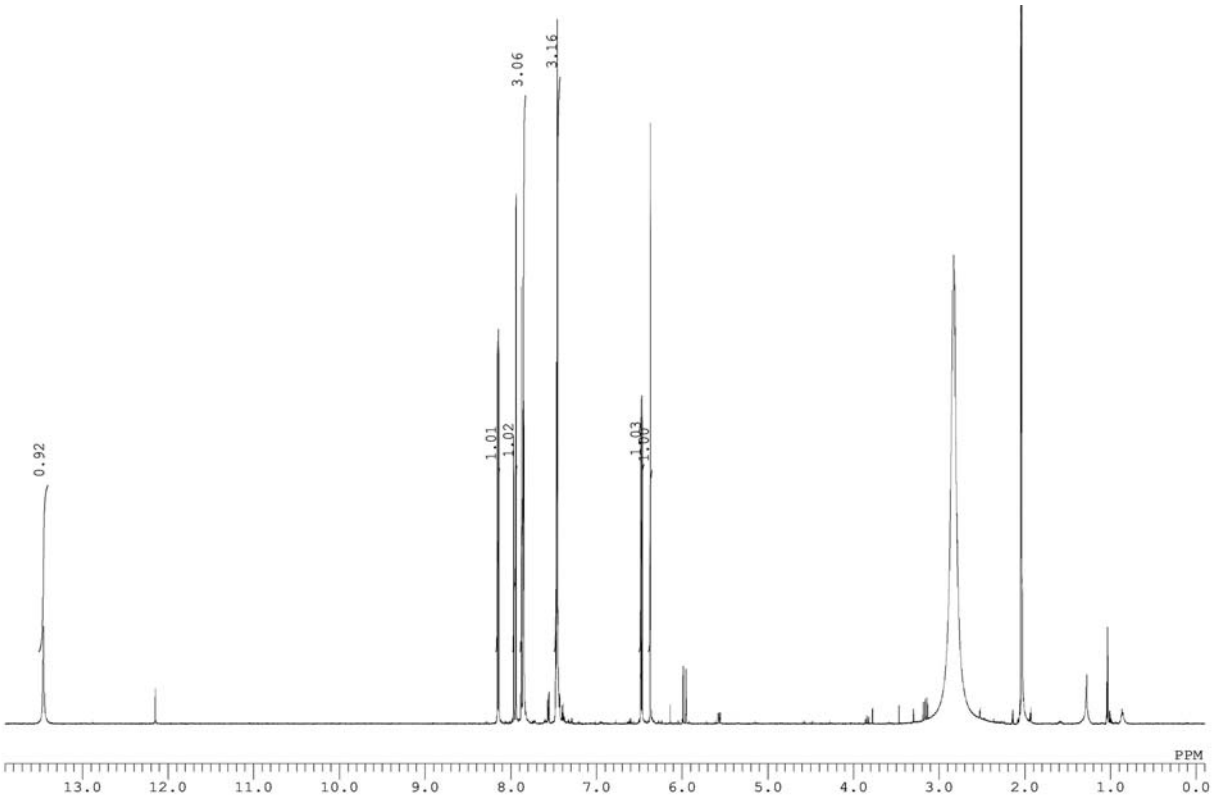
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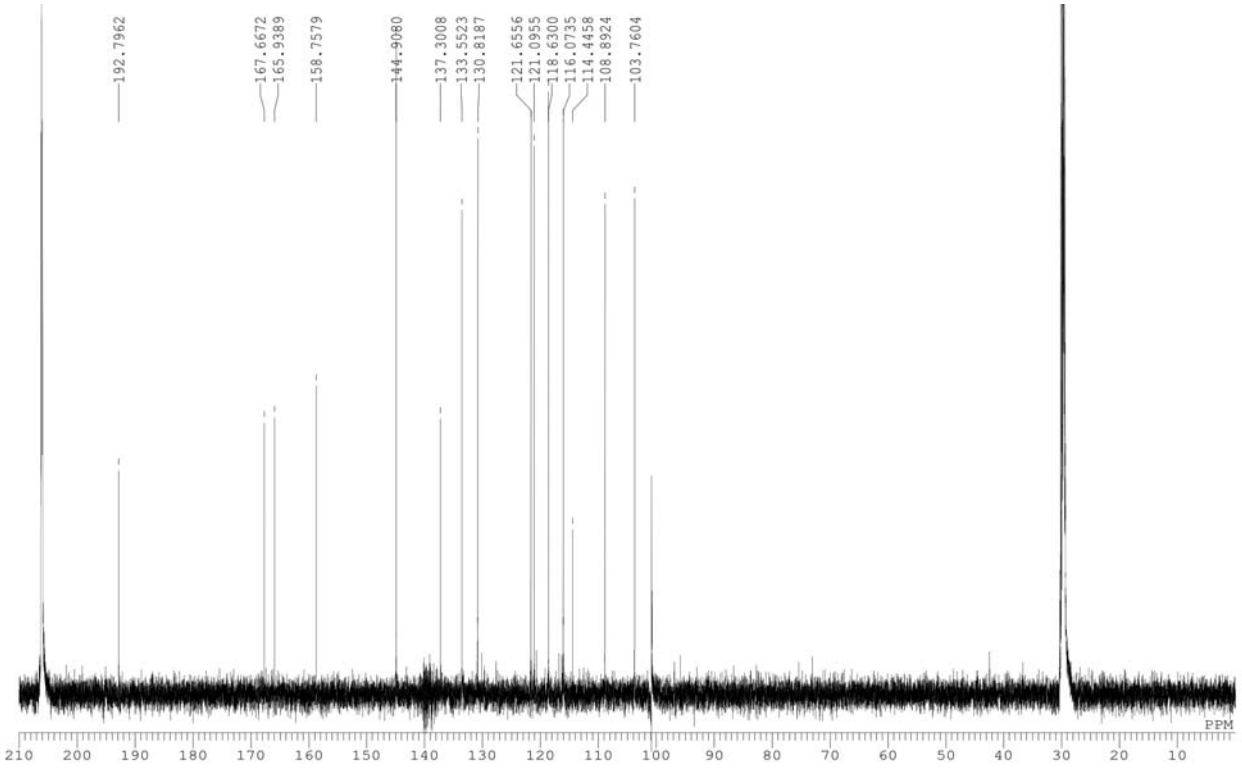
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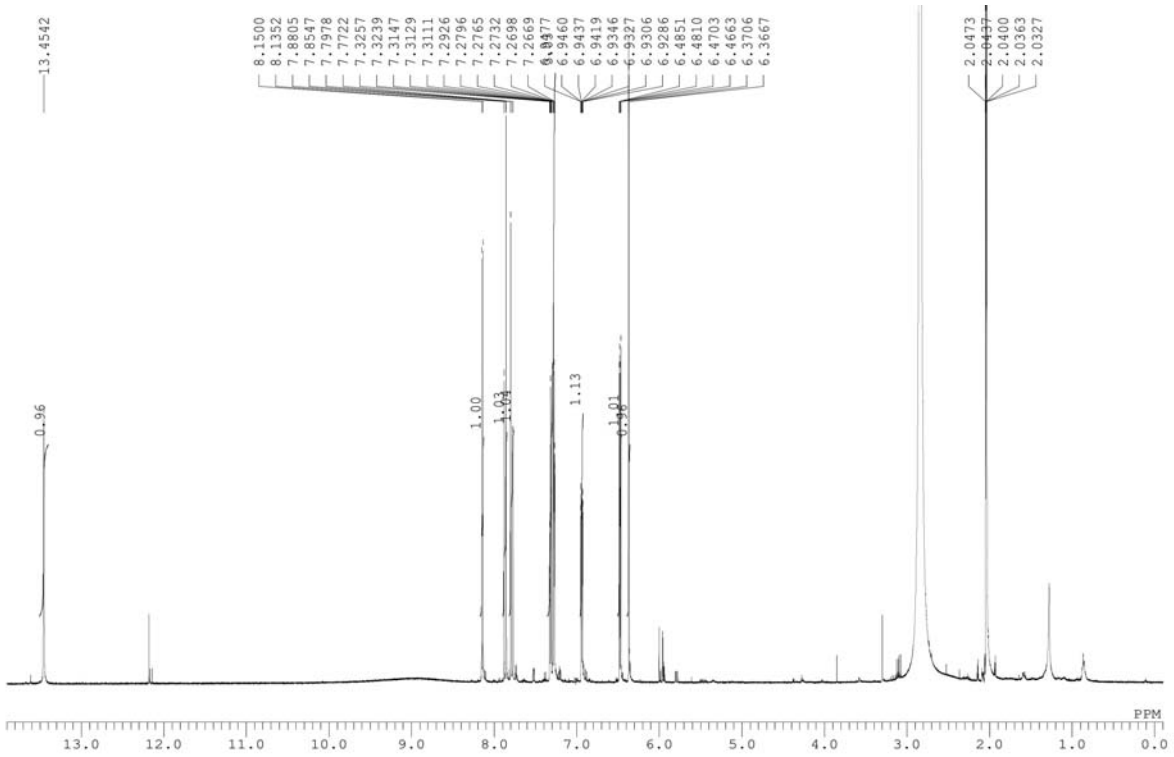
(e)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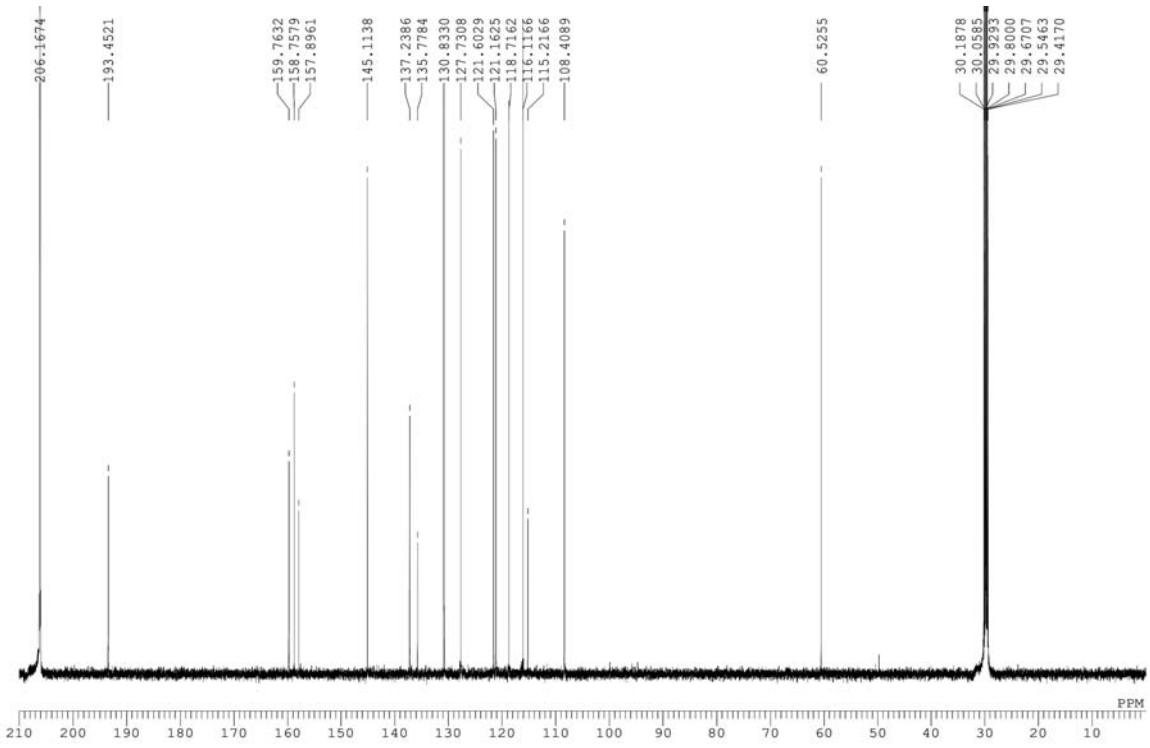
(f)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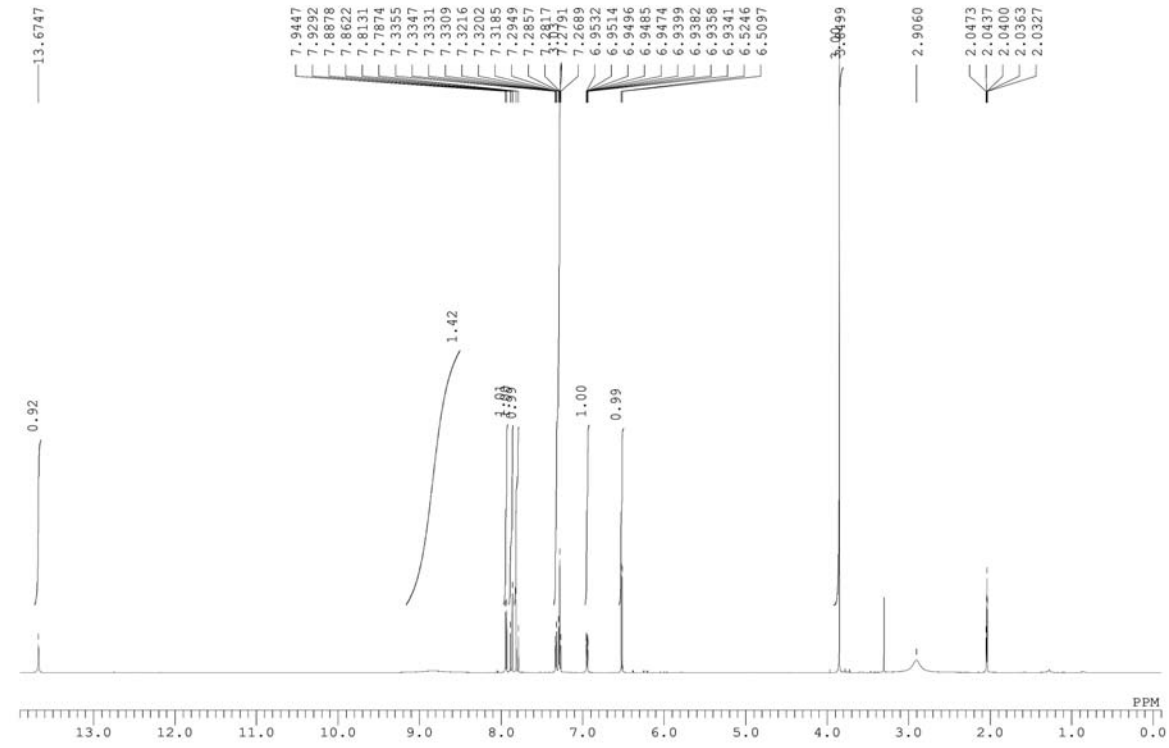
(g)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(h)

Figure 6.6 NMR spectrums of flavonoids isolated from the ethanol extract of *G. africana*

(a) ^{13}C NMR of (2*S*)-5,7,2'-trihydroxyflavanone

(b) ^{1}H NMR of (2*S*)-5,7,2'-trihydroxyflavanone

(c) ^{13}C NMR of (*E*)-2',4'-dihydroxychalcone

(d) ^{1}H NMR of (*E*)-2',4'-dihydroxychalcone

(e) ^{13}C NMR of (*E*)-3,2',4'-trihydroxychalcone

(f) ^{1}H NMR of (*E*)-3,2',4'-trihydroxychalcone

(g) ^{13}C NMR of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

(h) ^{1}H NMR of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

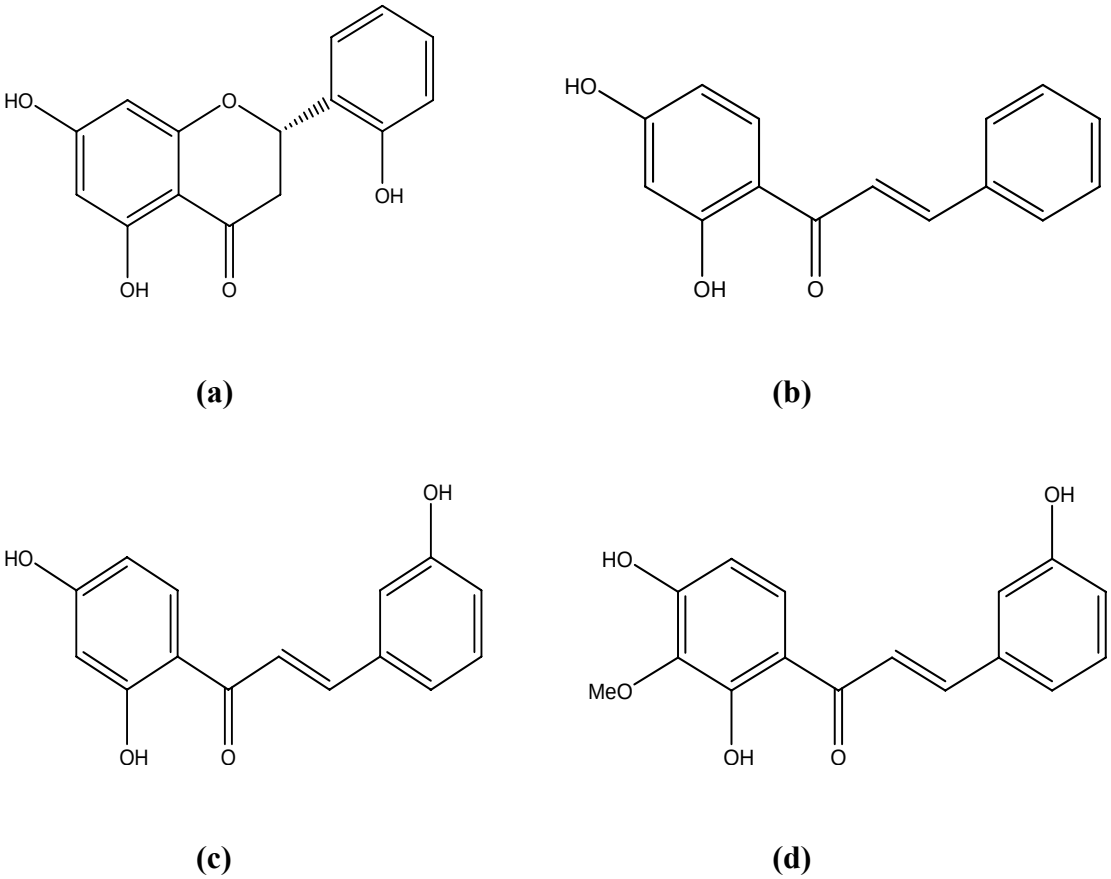


Figure 6.7. **Chemical structures of isolated compounds from the ethanol extract of *G. africana***

(a) (2*S*)-5,7,2'-trihydroxyflavanone

(b) (*E*)-2',4'-dihydroxychalcone

(c) (*E*)-3,2',4'-trihydroxychalcone

(d) (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

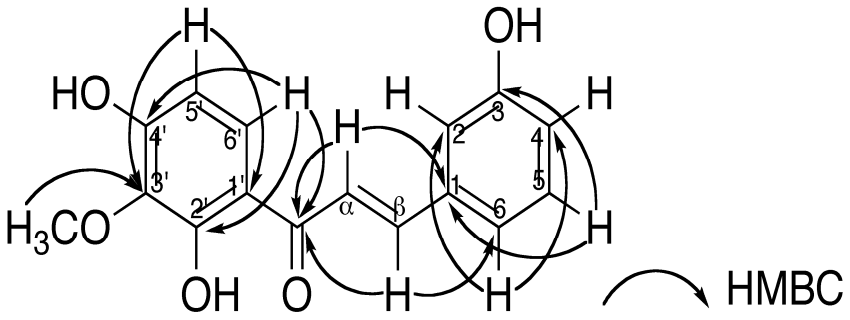


Figure 6.8. The HMBC spectrum of (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone

6.4. Conclusion

Nature has a proven ability to produce diverse and novel chemical prototypes with potential for possible drugs. Natural products like medicinal plants, play an important role in the discovery of potential innovative leads in drug development and in the development of high quality herbal drugs with proven efficacy. Rapid detection of biologically active natural products play a key role in the phytochemical investigation of crude plant extracts.

In order to perform an efficient isolation of compounds from any crude plant extracts, different techniques are used, such as low pressure column chromatography, ion-exchange methods, mass spectrometry, HPLC, planar chromatography and high-speed countercurrent chromatography to provide numerous structural data for isolation (Bohlin, 1998).

Due to the good inhibitory activity of the ethanol extract of *G. africana* amongst the other selected plants, the ethanol extract was selected for the isolation of compounds. Three known and one novel compounds belonging to flavonoid and chalcone groups were isolated. These compounds are: (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-2',4'-dihydroxychalcone, (*E*)-3,2',4'-trihydroxychalcone and (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone. Two compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone were previously isolated

by other researchers. Compound, (*E*)-3,2',4'-trihydroxychalcone has not been reported from natural sources but previously isolated by Severi *et al*., 1998 and a novel compound, (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone is isolated from *G. africana* for the first time. Biological activity of some of the compounds isolated in the present study has been reported by other researchers previously.

Chalcones are a subset of compounds known as flavonoids. In a chalcone, two aromatic rings are joined by a 3-carbon unsaturated carbonyl system.

CHAPTER 7
DETERMINATION OF THE ANTIMYCOBACTERIAL ACTIVITY OF THE FRACTIONS AND ISOLATED COMPOUNDS FROM THE ROOTS OF *GALENIA AFRICANA* L. VAR. *AFRICANA*

7.1. Introduction

The escalation of infections caused by *M. tuberculosis*, particularly those caused by MDR-TB strains is of considerable global concern. Since no new antituberculosis drugs have become available during the last forty years, there is obvious need for new and effective anti-tubercular drugs. There are reports of phytochemical analysis of essential oils, glycolipids, sesquiterpenoids, triterpenoids terpenes, steroids, tannins and triterpenoid as having potential antimycobacterial activity (Rivero-Cruz *et al*., 2005, Redo *et al*., 2006, Mann *et al*., 2008,). Previous studies of isolated compounds such as naphthoquinones (diospyrin, isodiospyrin, mamegakinone, 7-methyljuglone, neodiospyrin and shinanolone) were found to have antimycobacterial properties (Lall, 2005). Prenylated xanthones (α- and β-Mangostins and garcinoneβ) isolated from *Garcinia mangostana*, were found to have strong antimycobacterial inhibitory effect (Suksamrarn *et al*., 2003).

In this study, antimycobacterial activity of the isolated compounds from fractions of the ethanol extract of the roots of *G. africana* are evaluated using two *Mycobacterium* species.

7.2. Materials and Methods

Fractionation of fractions and isolation of compounds from *G. africana* are described in Chapter 6. Ciprofloxacin and Isoniazid (INH) were purchased from Sigma-Aldrich, Johannesburg, South Africa. The drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) and *M. smegmatis* (Mc^2 155) were similar to the ones used previously (Chapter 4 and 7).

7.2.1. Microplate susceptibility testing against *M. smegmatis*

To test for antimycobacterial activity, the ethanol extract, Fractions I – VI and the isolated compounds from *G. africana*, were tested against *M. smegmatis* using the microplate dilution method described in Chapter 4, section 4.2.3.2. Each sample was dissolved in 10% DMSO in sterile Middlebrook 7H9 broth base to obtain a stock concentration of 100.0 mg/mL and 0.5 mg/mL respectively. Dilution series of the sequential extracts, Fractions I – VI and the isolated compounds to be evaluated were made with 7H9 broth to yield volumes of 100.0 µL/well with the final concentrations ranging from 25.0 to 0.195 mg/mL. Ciprofloxacin at a final concentration of 0.156 mg/mL, served as the positive drug control. The MIC and MBC were determined as described in Chapter 4, section 4.2.3.2.

7.2.2. Antitubercular rapid radiometric assay using *M. tuberculosis*

The radiometric respiratory system with the BACTEC apparatus was used for the susceptibility testing of *M. tuberculosis*. Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A drug-susceptible strain of *M. tuberculosis*, H37Rv obtained from American Type, MD.USA Culture Collection (ATCC), 27294, was used in the screening procedure. Each sample was dissolved at 10.0 mg/mL in DMSO and added to 4.0 mL of BACTEC 12B broth to achieve the final concentrations ranging from 5.0 – 0.05 mg/mL (in triplicates, one with PANTA, and two without PANTA, an

antimicrobial supplement). The BACTEC drug susceptibility testing was also done for the primary drug INH at concentration of 0.2 µg/mL against the H37Rv sensitive strain. Preparation of bacterial cultures and the testing procedures were the same as described in Chapter 4, section 4.2.3.4.

7.2.3. Direct bioassay

Due to insufficient amounts of the isolated compounds, only one compound, (2*S*)-5,7,2'-trihydroxyflavanone (40.0 µg/mL), was evaluated on TLC plates by applying a small spot of 20.0 µL to silica gel 60 PF_{254} plates. The plates was developed in $CH_3(CH_2)_4CH_3$: EtOAc (8:2) and dried carefully (Figure 7.2a). The 24 hour *M. smegmatis* (1.26 x10^8 CFU/mL) in 7H9 broth was centrifuged at 1000 rpm for 15 minutes. The supernant was discarded and the pellet was resuspended in fresh 7H9 broth. A fine spray was then used to apply the bacterial suspension onto the TLC plates (Dilika and Meyer, 1996). The plates were then incubated at 37^{o}C for 24 hours in humid conditions. After incubating, the plates were sprayed with 2.0 mg/mL INT and the inhibition zones were noted.

7.3. Results and Discussion

In the present study, the ethanol extract, fractions and the compounds were found to have inhibitory activity. The ethanol extract of *G. africana* was found to be active at 0.78 mg/mL and 1.2 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively. *G. africana* had an MBC of 1.56 mg/mL against *M. smegmatis* (Table 7.1). Fractions II - V showed activity and Fraction IV was the most active fraction exhibiting an MIC of 0.12 and 0.5 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively. Fraction IV also showed bactericidal properties with an MBC of 0.06 mg/mL. The antituberculosis activity of (2*S*)-5,7,2'-trihydroxyflavanone showed activity at 0.03 and 0.1 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively. (2*S*)-5,7,2'-trihydroxyflavanone showed bactericidal effects at 0.12 mg/mL against *M. smegmatis* (Table 7.1).

Table 7.1. Antimycobacterial activity of the ethanol extract, fractions and isolated compounds from *G. africana* leaves against *M. smegmatis* and *M. tuberculosis*

Tested samples	***M. smegmatis***		***M. tuberculosis***	
	MIC[a] (mg/mL)	MBC[b] (mg/mL)	MIC (mg/mL)	ΔGI[c]
Ethanol extract of *G. africana*	0.78	1.56	1.20 (S[d])	0.0 ± 0.0
Fraction I	na[e]	nt[f]	na[g]	227.5 ± 314.6
Fraction II	0.50	na	na	200.5 ± 122.3
Fraction III	0.25	na	1.00 (S)	18.5 ± 4.9
Fraction IV	0.12	0.06	0.50 (S)	20.0 ± 1.4
Fraction V	0.25	na	1.00 (S)	28.0 ± 5.6
Fraction VI	na	nt	na	35.5 ± 6.3
(2*S*)-5,7,2'-trihydroxyflavanone	0.03	0.12	0.10 (S)	8.0 ± 2.8
(*E*)-2',4'-dihydroxychalcone	0.12	0.06	0.05 (S)	2.0 ± 1.4
(*E*)-3,2',4'-trihydroxychalcone	na[h]	nt	0.10 (S)	2.0 ± 1.4
(*E*)-3,2',4'-trihydroxy-3'-methoxychalcone **(Novel)**	na	nt	0.05 (S)	19.0 ± 2.6
Ciprofloxacin (positive drug control for *M. smegmatis*)	0.15	0.31	nd[i]	nd
Isoniazid (positive drug control for *M. tuberculosis*)	nd	nd	2×10^{-4} (S)	13.0 ± 0.7

[a]Minimum inhibitory concentration.

[b]Minimum bactericidal concentration.

[c]ΔGI value (mean ± SD) of the control vial (10^{-2}), 38.0 ± 3.8 for the sensitive strain.

[d]Susceptible.

[e]Not active at the highest concentration tested (6.25 mg/mL).

[f]Not tested for MBC determination.

[g]Not active at highest concentration tested (200.0 mg/mL).

[h]Not active at the highest concentration tested (0.62 mg/mL).

[i]Not determined.

The other compound, (*E*)-2',4'-dihydroxychalcone showed inhibitory activity at 0.12 and 0.05 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively, with MBC of 0.06 mg/mL against *M. smegmatis* (Table 7.1).

(2*S*)-5,7,2'-trihydroxyflavanone previously isolated by Miyaichi *et al*., 2006 from the roots of *Scutellaria amabilis* has been reported to have anticancer and anti-inflammatory activity. Other researchers (Vries *et al*., 2005; Zampini *et al*., 2005; Svetaz *et al*., 2007), isolated (*E*)-2',4'-dihydroxychalcone from crude extracts of *G. africana* and *Zuccagnia punctata* which has been reported to have antifungal and antibacterial activity. Su *et al*., 2003 isolated (*E*)-2',4'-dihydroxychalcone from *Muntingia calabura* and tested for an *in vitro* quinone reductase induction assay. The other compounds, (*E*)-3,2',4'-trihydroxychalcone (not reported from natural sources but previously isolated by Severi *et al*., 1998 and (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone (novel compound) are isolated from *G. africana* for the first time.

In another study, alpinumisoflavone, genistein (5,7,4'-trihydroxyisoflavone), laburnetin and luteolin (3',4',5,7-tetrahydroxyflavone), were valuated against *M. smegmatis* and *M. tuberculosis*. Laburnetin showed the best activity against *M. tuberculosis* exhibiting an MIC of 4.88 µg/mL (Kuete *et al*., 2008).

Lin *et al* (2002), evaluated a series of flavonols (3-hydroxyflavones), flavonones, chalcones (1,3-diarly-2-propen-1-ones) and chalcone-like compounds (1,3-heteroaromatic ring-substituted 2-propen-1-ones) for inhibitory activity against *M. tuberculosis*. Chalcones with a 2-hydroxyl group: 1-(2-hydroxyphenyl)-3-(3-chlorophenyl)-2-propen-1-one and 1-(2-hydroxyphenyl)-3-(3-iodophenyl)-2-propen-1-one, demonstrated 92% inhibition at 12.5 µg/mL. Chalcone-like compounds (heterocyclic ringsubstituted 2-propen-1-one): 1-(4-fluorophenyl)-3-(pyridin-3-yl)-2-propen-1-one, 1-(3-hydroxyphenyl)-3-(phenanthren-9-yl)-2-propen-1-one, 1-(pyridin-3-yl)-3-(phenanthen-9-yl)-2-propen-1-one and 1-(furan-2-yl)-3-phenyl-2-propen-1-one, exhibited 96 - 98% inhibition at 12.5 µg/mL. All tested flavones and flavanones were found to have moderate to weak antimycobacterial activity.

Chapter 7 *Antimycobacterial activity of the fractions and isolated compounds*

In vitro studies of novel chalcones synthesised by condensing 2-(4-carboxyphenylazo) acetoacetate, were screened for their antimycobacterial activity against *M. tuberculosis* H37Rv using a BACTEC-460 radiometric system. The compounds exhibited >90% inhibition against *M. tuberculosis* at 6.25 µg/mL. This antimycobacterial activity indicated that the presence of 2-nitro, 3-nitro and 4-methoxy substitution on chalcone produced remarkable improvements in antitubercular activity (Trivedi *et al*., 2008). A few compounds belonging to flavones, flavonones and chalcones showed comparatively better activity than the compounds tested in the present study.

The bactericidal effect of the ethanol extract, fractions, purified compounds and INH in the BACTEC system, was compared between the treated and untreated cultures. 100.0 µL of the bacterial suspensions from BACTEC vials (exhibiting MICs) at the end of the experiment were plated on 7H11 agar medium for viable count enumeration. Only selected results (expressed as mean viable counts ± standard error) in case of the treated and untreated vials are illustrated in figure 7.1. Fractions IV resulted in 1 log (90%) killing at 0.5 mg/mL. The four isolated compounds were more bactericidal than the crude ethanol extract and resulted in a 2 log (99.5%) killing at 0.1 to 0.05 mg/mL (Figure 7.1).

In a bioautographic assay, (2*S*)-5,7,2'-trihydroxyflavanone sprayed with INT and reincubated at 37^{o}C for 4 hours, the plates changed colour to a reddish-pink where bacterial growth occurred and a zone of inhibition by the compound was observed (Dunigan *et al*., 1995; Figure 7.2b).

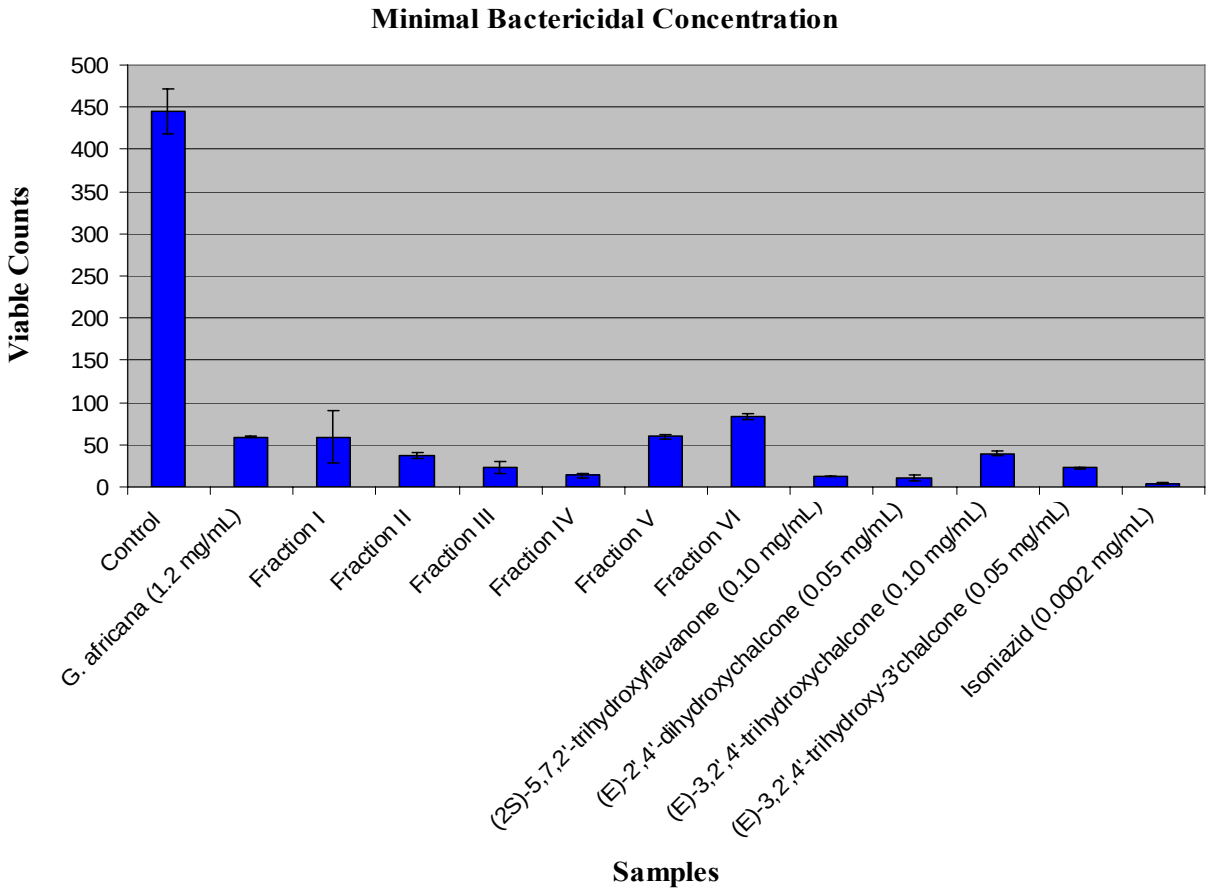
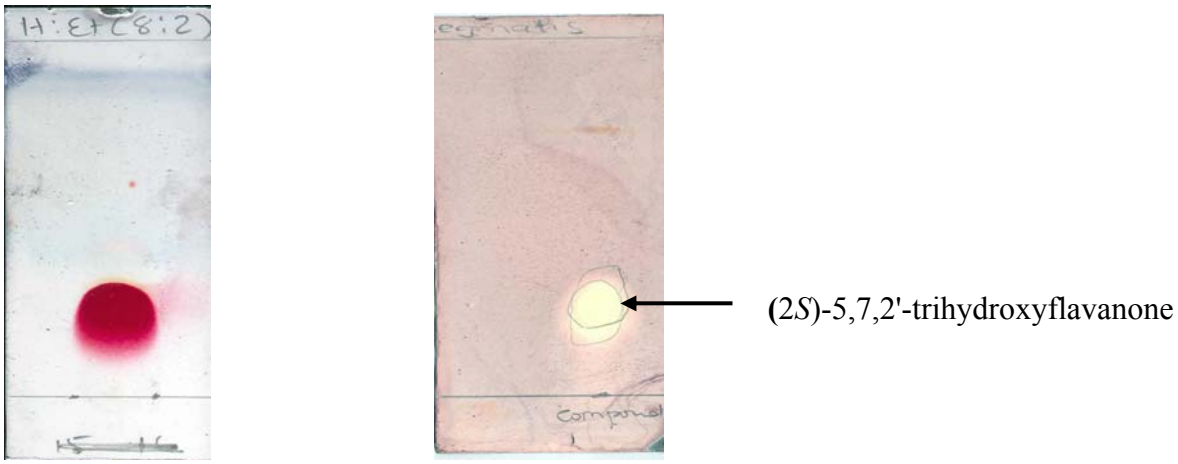


Figure 7.1. The comparative bactericidal effect of the ethanol extract, fractions and isolated compounds of *G. africana* against the drug-susceptibility strain of *M. tuberculosis*. Results illustrate the mean of the viable bacterial counts ± standard error in the treated vials as compared to the untreated control vials.



(a) (b)

Figure 7.2. Zones of inhibition of *M. smegmatis* on TLC plates in a direct assay of (2*S*)-5,7,2'-trihydroxyflavanone isolated from the *G. africana* ethanol extract. Solvent systems: hexane: ethyl acetate (8:2)
(a) compound not sprayed with *M. smegmatis*
(b) compound sprayed with *M. smegmatis*

7.4. Conclusion

This is the first report of the antituberculosis activity of ethanol extract of *G. africana* and its isolated compounds against *M. tuberculosis*.

Our findings indicated some correlation between the activities of the ethanol extract of *G. africana* with its constituents when screened against both *M. smegmatis* and *M. tuberculosis*. Selection of plants by ethnobotanical criteria offers a good probability of finding candidates which contain compounds active against mycobacteria (Lall and Meyer, 1999).

CHAPTER 8
SYNERGISTIC EFFECT, CYTOTOXICITY AND INTRACELLULAR ACTIVITY AGAINST *M. TUBERCULOSIS*

8.1. Introduction

The recent upsurge in the incidence of TB with the significant emergence of multidrug-resistant cases has focused on the priority of discovering effective new drugs and on the strategies to augment the potential of existing drugs against *M. tuberculosis*. *M. tuberculosis* is a complex, resilient organism, and it is important to recognize that new, better TB treatments will still require drugs to be taken in combination, in order to reduce TB's six month treatment time, be effective against drug-resistant strains, and be compatible with anti-retroviral therapy used to treat patients with TB-HIV. One of the major components of the strategic plan to eliminate TB is the use of antimycobacterial drugs to destroy *M. tuberculosis* residing in the body. INH has been the drug choice for over 30 years in treating a quiescent condition and in prophylaxis therapy, however, long-term therapy results in hepatitis. This, coupled with the emergence of INH-resistant TB, has led to an increasing need for alternative preventive-therapy regimens (American Thoraic Society. 1986, Ferebee, 1970).

No new TB drugs have been discovered in the last 40 years and emerging strains of the bacterium, that are resistant to multiple drugs, are increasing. Current therapy for drug-sensitive TB recommended by WHO consists of a cocktail of four drugs (INH, RIF, PZA and EMB), taken for six to nine months. The first-line drugs are cheap and have few side effects. Second-line or third-line drugs are more expensive, less potent and some are as toxic as cancer chemotherapy and require hospitalization.

MDR-TB treatment using currently available second-line drugs may cure only 65% - 75% of patients (Mukherjee *et al*., 2004). Drug-resistant TB is "humanmade": it results from treatment with inadequate drugs or drug regimens, improper case management and preventable transmission. New drugs are badly needed that can outmatch mutated strains and shorten and simplify treatment.

Since the main route of entry of TB is the respiratory route, alveolar macrophages are the important cell types, which combat the pathogen (Alamelu, 2004). The initial step in the infection process of TB is inhaling the bacteria which is readily phagocytosed, processed and presented by alveolar macrophages to the T-lymphocytes (Hockings and Golde, 1979). *M. tuberculosis* is an obligatory aerobic, intracellular pathogen that resides predominantly within macrophages. Macrophages are leukocytes cells within the tissues that originate from specific white blood cells. Macrophages develop from bone marrow precursors which mature and enter the bloodstream as monocytes. When a monocyte enters a damaged tissue through a blood vessel, it undergoes a series of changes to become a macrophage. The main role of the macrophage is the removal of necrotic debris (unusual death of cells and living tissues) and dust in the lungs. The removal of dust and necrotic tissue is to a greater extent handled by fixed macrophages, which stay in strategic locations such as the lungs, liver, neural tissue, bone, spleen and connective tissue, ingesting foreign materials such as dust and pathogens, calling upon wandering macrophages if needed. When a macrophage ingests a pathogen, it will present an antigen of the pathogen to a corresponding helper T cell. The antigen presentation results in the production of antibodies that attach to the antigens of the pathogens, making it easier for the macrophages to adhere to their cell membrane and phagocytose. The pathogen becomes trapped in a food vacuole, which then fuses with a lysosome. Within the lysosome, enzymes and toxic peroxides digest the invader. However, some bacteria such as *M. tuberculosis* have become resistant to these methods of digestion (Fenton, 1998).

The entry of *M. tuberculosis* into the host macrophage is the key component of TB pathogenesis. Phagocytosis of *M. tuberculosis* by alveolar macrophages is the first event in the host-pathogen relationship that decides outcome of infection. Within 2 to

6 week of infection, cell-mediated immunity (CMI) develops, and there is an influx of lymphocytes and activated macrophages into the lesion resulting in granuloma formation. The exponential growth of the bacilli is checked and dead macrophages form a caseum. The bacilli are contained in the caseous centers of the granuloma. The bacilli may remain forever within the granuloma, or get re-activated later and may get discharged into the airways after enormous increases in numbers causing necrosis of bronchi and cavitation. Fibrosis represents the last-ditch defense mechanism of the host, and is the surrounding of the central area of necrosis to wall off the infection when all other mechanisms have failed (Alamelu, 2004). In order for *M. tuberculosis* to bind on monocytes macrophages, the complement receptors (CRl, CR2, CR3 and CR4), mannose receptors (MR) and other cell surface receptor molecules play an important role in the binding of the organisms to the phagocytes (Schlesinger, 1994). The interaction between MR on phagocytic cells and mycobacteria seems to be mediated through the mycobacterial surface glycoprotein lipoarabinomannan (LAM). Prostaglandin E2 (PGE2) and interleukin (IL)-4 (a Th2-type cytokine), regulate CR and MR receptor expression, and the interferon-Î³ (IFN-Î³) decreases the receptor expression, resulting in diminished ability of the mycobacteria to adhere to macrophages (Barnes *et al*., 1994). Surfactant protein receptors, CD14 receptor and the scavenger receptors also have a role in mediating bacterial binding (Gaynor *et al*., 1995). Novel approaches to therapy and new drugs are urgently needed in order to act within the host macrophages and to have direct access to the dormant organisms that presumably are within the macrophages (Quenelle *et al*., 2001).

The chemotherapy of pathogenic organisms relies on metabolic differences between pathogens and their mammalian hosts. A possible mode of action in *M. tuberculosis*, is the presence of thiols. The thiol compounds such as: cysteine, coenzyme A, gluthathione, lipoamide, transgluthaminase methanethiol, mycothione, play an important role in maintaining thiol groups, required for the activity of many enzymes in a reduced state and serve as cofactors for a number of enzymes involved in the detoxification and export of toxic compounds from cells. *M. tuberculosis* lacks glutathione, but instead maintains millimolar concentrations of the structurally distinct low molecular weight thiol mycothiol (MSH, Figure 8.1), found in actinomycetes,

mycobacteria and streptomycetes. MSH is comprised of N-acetylcysteine amide-linked to GlcN-alpha (1-1)-Ins (Newton and Fahey., 2002). In *M. tuberculosis*, MSH protects the bacteria from toxic oxidants and antibiotics. MSH-deficient mycobacteria exhibit increased sensitivity to oxidative stress, making this redox pathway a potential biological target for novel antitubercular chemotherapies (Patel and Blanchard, 1999). Many compounds like napthoquinones are known to operate as subversive substrates with flavoprotein disulfide reductases such as gluthathione reductase, trypanothione reductase and lipoamide dehydrogenase (Biot *et al*., 2004., Salmon-Chemin *et al*., 2001). The functions of these enzymes involve the NAD(P)H-dependent reduction of disulfide bonds in proteins or oxidised version of low molecular weight thiols such as glutathione. The NADPH-dependent enzyme mycothiol disulfide reductase (Mtr, Figure 8.2) helps to maintain an intracellular reducing environment by reducing MSSM back to MSH (Spies *et al*., 1994). Since there is no mammalian counterpart to the pathway it should be possible to achieve a selective inhibition of mycothiol biosynthesis. Enzymes involved in the biosynthesis of mycothiol could make attractive drug targets and another possibility is to reduce the effect of endogenous mycothiol to inhibit enzymes involved in degrading the mycothiol-antibiotic complex (Newton *et al*., 2000).

In the present study, we investigated the synergistic activity of only two isolated compounds from the roots of *G. africana* (same as in Chapter 7) in combination with INH against *M. tuberculosis*. The ethanol extract, and the two isolated compounds were also evaluated against the TB infected human macrophage cell line. The biological importance of Mtr, with its ability to turnover antimycobacterial compounds as subversive substrates and also to provide an insight into possible mode of action as properties with *M. tuberculosis* Mtr is included.

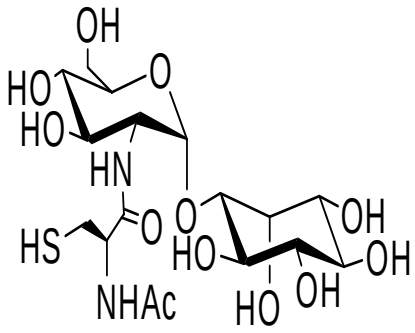


Figure 8.1. Chemical structure of mycothiol (MSH)

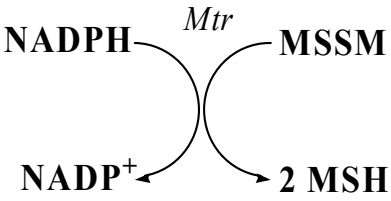


Figure 8.2. Structure of mycothiol disulfide reductase (Mtr)

8.2. Materials and Methods

The drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) was similar to the one used previously (Chapter 4 and 7). INH (Sigma-Aldrich, South Africa) was purchased in a powder form. Due to insufficient amounts of the compounds, only two compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone isolated from the roots of *G. africana*, as described in Chapter 6, were tested.

8.2.1. MIC determination: combination drug-action

MIC's of the test compounds and INH were determined by the radiometric method as described in Chapter 4 and 7. Final concentrations of each test compounds ranged in a two-fold dilution from 100.0 to 6.25 µg/mL and for INH from 0.2 to 0.0125 µg/mL.

8.2.1.1. Combined drug action by the radiometric method

The activity of the individual drugs and the two-drug combination were evaluated at sub-MIC levels so that each compound were present at concentrations corresponding to 1/2; 1/4; 1/8; 1/16 and 1/32 of the documented MIC. Analysis of the drug combination data was achieved by calculating the fractional inhibitory concentration (FIC) index as follows: FIC = (MIC $_{\text{a combination}}$/ MIC $_{\text{a alone}}$) + (MIC $_{\text{b combin}}$ation + MIC $_{\text{b alone}}$). The FIC was interpreted as: FIC $\leq$ 0.5, synergistic activity; FIC = 1, indifference / additive activity; FIC $\geq$ 2 or more, antagonistic activity. Subscripts a and b represent the two different compounds (Bapela *et al*., 2006; Berenbaum 1978; De Logu *et a*l., 2002).

8.2.2. Cell line

Culture of human monocytes, U937, displaying macrophage-like activity, were cultured in RPMI (developed at Roswell Park Memorial Institute) 1640 medium (pH 7.2 Sterilab, South Africa), supplemented with 10% fetal bovine serum and 2 mM_L-glutamine and with antibiotics-pennicilin, streptomycin and fungizone solution (0.1%). Cells were grown to a density of 5 $x10^8$ cells/mL (the cells were grown till confluent-thus till the concentration of the cells were very high), centrifuged and washed with phosphate buffered saline solution. The cells were then counted with a hematocytometer using a light microscope to determine the concentration of the cells and resuspended in the correct amount of supplemented RPMI 1640 medium as calculated and resuspended in supplemented RPMI 1640 medium. The concentration of cells was thus adjusted to 10^5 cells/mL. At first 1.0 uL of TPA (12-o-tetradecanoyl 13-acetate) DMSO solution was added to 10.0 mL of complete medium, then 20.0 uL of the solution was added to all of the wells receiving the cells and finally 180.0 uL of cell suspension was added to each well of the 96-well tissue culture plate and incubated for 24 hours to stimulate maturation of the monocytes (Passmore *et al*., 2001; Hosoya and Marunouchi, 1992).

8.2.2.1. Cytotoxicity: U937 cell line

Cytotoxicity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide (XTT) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). 100.0 µL of U937 cell lines (1 x 10^5 cells/mL) were seeded into the inner wells of a microtiter plate, while in the outer wells 200.0 µl of incomplete medium was added. The plates were incubated for 24 hours to allow the cells to attach to the bottom of the plate. Dilution series were made of the ethanol extract of *G. africana* at the various concentrations (400.0 to 3.125 µg/mL) and for (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone, at 100.0 to 1.563 µg/mL. These dilutions were added to the inner wells of the microtiter plate and incubated for 72 hours. After 72 hours, 50.0 µL of XTT reagent (1.0 mg/mL XTT with 0.383 mg/mL PBS) was added to the wells and the plates were then incubated for 1-2 hours. The positive control, (Doxorubin), at final concentration ranging from 0.5 - 200.0 µg/mL, was included. After incubation, the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader, which measures the OD at 450 nm with a reference wavelength of 690 nm. DMSO was added to serve as the control for cell survival. The 'GraphPad Prism 4', statistical program was used to analyse the 50% inhibitory concentration (IC_{50}) values.

8.2.2.2. MIC determination: U937 cell line

The ethanol extract and each compound were dissolved at 20.0 mg/mL and 10.0 mg/mL in DMSO respectively, diluted further in complete RPMI 1640 medium to obtain concentrations that were 10 x higher than the final concentration required in the wells.

The final concentrations of the ethanol extract ranged from 0.1 to 0.025 mg/mL and for the test compounds from 0.4 to 0.010 mg/mL. The primary drug INH at concentrations of 0.6 and 0.4 µg/mL was included. The highest concentration of DMSO in the wells for testing against *M. tuberculosis* was 1% (Bapela, 2005).

8.2.2.3. Infection of cells

The cells were first washed with PBS three times, to remove extra-cellular bacteria and then infected with tubercle bacilli at a ratio of 10 to 20 bacilli per cell. The macrophages were allowed to phagocytize the bacteria for 4 hours at 37^0C. Then the number of intracellular organisms was determined by lysing the macrophages with 0.25% (w/v) sodium dodecyl sulfate (SDS), doing serial dilutions and plating the lysate on 7H11 agar medium for viable count determinations. After phagocytosis, 100.0 µL of the fresh medium containing the desired antimycobacterial agents (extract and compounds) was refed to each macrophage containing well, and the culture was enumerated after lysing the macrophages on day 5. After 5 days of incubation, the contents of each well were resuspended with a needle and syringe, and 100.0 µL was transferred to a degassed 4.0 mL BACTEC 12B vial. The vials were incubated at 37^0C overnight. The change in GI was recorded daily until the vials containing the 1:100 dilution of the untreated control suspension reached a GI of 30 or more. Interpretation of the results was the same as in Chapter 4, section 4.2.3.4. The MBC of the tested samples was assessed by plating the bacterial suspensions from the BACTEC vials which exhibited MIC, at the end of the experiment, on 7H11 agar medium for viable count enumeration (Rastogi *et al*., 1991).

8.2.3. Subversive substrate properties with mycothiol reductase

It was decided to investigate the effect of one compound, (2*S*)-5,7,2'-trihydroxyflavanone on mycothiol reductase. Other isolated compounds were insufficient. At our department a group of naphthoquinones have been identified with potent antutuberculosis activity (Lall and Meyer., 2001). Since naphthoquinones are known to operate as subversive substrates with flavoprotein disulfide reductase such as glutathione reductase, trypanothione reductase and lipoamide dehydrogenase (Biot *et al*., 2004., Salmon-Chemin *et al*., 2001), it was therefore, decided to include these naphthoquinones also in this study in order to compare with the compound '(2*S*)-5,7,2'-trihydroxyflavanon' isolated from *G. africana*. The derivatives of naphthoquinones used in this study were kindly donated by Dr Anita Mahapatra and

Dr Frank van der Kooy (Table 8.1). The enzyme-mediated toxicity of quinones or naphthoquinones is a consequence of their enzymatic reduction to semiquinone radicals. The naphthoquinone is then regenerated via the concomitant reduction of oxygen to toxic superoxide anion radicals. In this manner the naphthoquinone substrate is regenerated and the futile redox cycle continues (Figure 8.3). It seems plausible that some of these naphthoquinones could be exerting their biological activity as subversive substrates with similar disulfide reductases found in *M. tuberculosis*. Due to the availability of this '(2*S*)-5,7,2'-trihydroxyflavanone' only, one compound was included in this study.

Recombinant *M. tuberculosis* mycothione reductase (Mtr) was purified from *M. smegmatis* MC^2155 transformant (a generous gift from J. Blanchard) as previously described (Patel *et al*., 1999). Only (2*S*)-5,7,2'-trihydroxyflavanone was tested**.** Subversive substrate assays of (2*S*)-5,7,2'-trihydroxyflavanone with Mtr were carried out at 30°C in 1 cm^3 of 50 mM HEPES (pH 7.6), 0.1 mM EDTA containing Mtr (30.0 µg), NADPH (70.0 µM), and varying concentrations of substrate. Mtr was pre-incubated with NADPH for 5 min at 30˚C before initiating the reaction by addition of a DMSO solution of (2*S*)-5,7,2'-trihydroxyflavanone. The final DMSO concentration in the assays was (2 % (v/v)). Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH consumption.

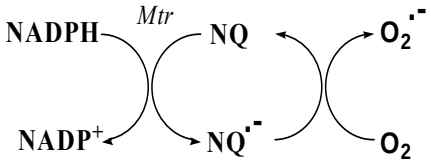
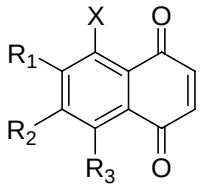
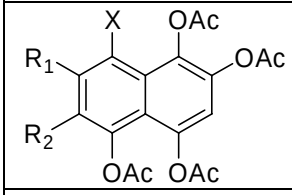
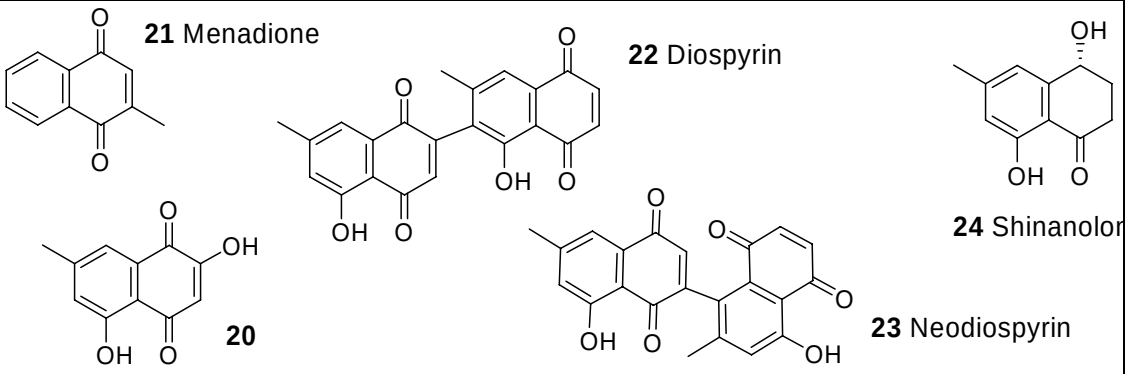


Figure 8.3. Enzymatic reduction of naphthoquinone to semiquinone

Table 8.1 List of naphthoquinones studied for disulfide reductase activity

Structure	NQ	X	R_1	R_2	R_3
	**1**	H	H	H	OH
	2	F	Me	H	OH
	3	Cl	Me	H	OH
	4	Br	Me	H	OH
	5	Cl	H	Me	OH
	6	H	Me	H	OH
	7	H	H	Me	OH
	8	H	Me	H	OAc
	9	Cl	Me	H	OAc
	10	Cl	H	Me	OAc
	11	H	Me	H	OMe
	12	Cl	Me	H	OMe
	13	Cl	H	Me	OMe
	14	H	Me	H	OEt
	15	Cl	Me	H	OEt
	16	Cl	H	Me	OEt
	**17**	H	Me	H	
	18	Cl	Me	H	
	19	Cl	H	Me	



8.3. Results and Discussion

8.3.1. Combination drug action

The development of antibiotic resistance can be natural or acquired and this can be transmitted within same or different species of bacteria. Natural resistance is

achieved by spontaneous gene mutation and the acquired resistance is through the transfer of DNA fragments like transposons from one bacterium to another. Bacteria gains antibiotic resistance due to three reasons namely: (i) modification of active site of the target resulting in reduction in the efficiency of binding of the drug, (ii) direct destruction or modification of the antibiotic by enzymes produced by the organism or, (iii) efflux of antibiotic from the cell (Sheldon, 2005). One strategy employed to overcome these resistance mechanisms is the use of combination of drugs. Few new agents are in development today for treating TB, and none has been designed specifically to shorten the treatment regimen. Drug design targeting the latency stage and synergistic interaction between the various drug candidates might prove to be good alternatives (Shanmugam *et al*.,2008).

The MICs and FICs of compounds alone and the combination effect against *M. tuberculosis* are shown in Table 8.2. The MICs of the ethanol extract, compounds and INH, which were previously reported in Chapter 7 were still the same (Table 7.1). The combination drug action showed that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone have synergistic action. Combination of (2*S*)-5,7,2' trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone reduced their original MICs four-fold resulting in an FIC of 0.5 indicating synergistic activity. The most pronounced effect of the two-drug action was demonstrated by both compounds with INH. This combination reduced their MICs sixteen-fold. The FIC index of (2*S*)-5,7,2'-trihydroxyflavanone and INH was 0.125 and for (*E*)-2',4'-dihydroxychalcone with INH was 0.18 indicating synergistic activity. The crude ethanol extracts from which the compounds were isolated, showed inhibitory activity at MIC of 1200.0 µg/mL, which suggests that there are other components within the extract which might have antimycobacterial effect. There have been no reports of similar flavonoids for combination action against *M. tuberculosis*. This is the first report on the synergistic effects of these compounds.

In another study, flavonoids (epicatechin, isorhamnetin, kaempferol, luteolin, myricetin, rutin, quercetin and taxifolin) were screened in combination with INH

Table 8.2. Synergistic effect and intracellular activity of the ethanol extract and isolated compounds against *M. tuberculosis*

Tested samples	*M. tuberculosis*				
	Combination drug action			Intracellular activity	
	MIC[a] (µg/mL)	ΔGI[b]	FIC[c]	MIC (µg/mL)	ΔGI[d]
Ethanol extract	1200.00 (S[e])	0.0 ± 0.0	nd[f]	50.0(S)	23.0 ± 16.2
(2*S*)-5,7,2'-trihydroxyflavanone	100.00 (S)	5.0 ± 3.5	nd	100.0 (S)	09.0 ± 06.3
(*E*)-2',4'-dihydroxychalcone	50.00 (S)	6.0 ± 4.2	nd	50.0 (S)	02.0 ± 01.4
INH	0.20 (S)	0.0 ± 0.0	nd	0.4 (S)	13.0 ± 09.4
(2*S*)-5,7,2'-trihydroxyflavanone + INH	1/16 +1/16 (S)	0.0 ± 0.0	0.1250	nd	nd
(*E*)-2',4'-dihydroxychalcone + INH	1/8 + 1/8 (S)	0.0 ± 0.0	0.1875	nd	nd
(2*S*)-5,7,2'-trihydroxyflavanone + (*E*)-2',4'-dihydroxychalcone	¼ + ¼ (S)	16.0 ± 11.3	0.5000	nd	nd

[a]Minimum inhibitory concentration.

[b]ΔGI value (mean ± SD) of the control vial (10^{-2}) was 32.0 ± 22.6 for the sensitive strain.

[c]Fractional inhibitory concentration.

[d]ΔGI value (mean ± SD) of the control vial (10^{-2}) was 42.0 ± 29.6 for the sensitive strain within macrophages.

[e]Susceptible.

[f]Not determined.

against different mycobacterial strains. The best synergistic activity was observed on myricetin which exhibited a synergistic and not additive antibiotic effect with INH (FIC of 0.2) by decreasing the MIC of INH 16-fold at a concentration of 8 µg/mL and 64-fold at a concentration of 16 µg/mL against *M. smegmatis* (Lechner *et al*., 2008). Bapela *et al* (2006), reported combination studies of 7-methyljuglone with INH and RIF. FIC indexes obtained were 0.2 and 0.5 for RIF and INH respectively, suggesting a synergistic interaction between 7-methyljuglone and these anti-TB drugs.

8.3.2. Intracellular activity

Effective doses of antimycobacterial drug should also be evaluated in a macrophage model to ensure intracellular drug effectiveness (Chanwong *et al*., 2007). *M. tuberculosis* infection is a complex process that initiates with aerosol inhalation to the lung, wherein; the mycobacteria are phagocytosed by alveolar macrophages. Upon entry into macrophage, the TB bacilli interfere with normal phagosomal maturation, preventing fusion with lysosomes. In response to this infection, macrophages produce pro-inflammatory signals (cytokines and chemokines) to recruit T-cells to the infected lung tissue which induces coughing and provides an exit strategy for the bacteria to spread to another host (Bhave *et al*., 2007).

Broad ranges of the biological activities of compounds have been reported using *in vitro* studies. The antimycobacterial activity of the ethanol extract of *G. africana*, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone against *M. tuberculosis* residing within U937 macrophage cells were investigated. The ethanol extract of *G. africana*, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone inhibited the growth of *M. tuberculosis* residing within macrophages at concentrations of 50.0; 100.0 and 50.0 µg/mL respectively (Table 8.2). This study indicates that the activity of the ethanol extract is more active in macrophages and that of the isolated compounds is still at their MIC values obtained in extracellular experiments (Chapter 7, Table 7.1). This might be an indication that the ethanol extract could be more taken up by macrophages or other molecules in the cell, leading to the increased interaction with the bacteria. In human macrophage

cultures, antituberculosis drugs have been found to have inhibitory effects on tubercle bacilli residing within cultured human macrophages. INH was found to have an MIC of 0.05 µg/mL against tubercle bacilli in macrophages. PZA was found to have a bacteriostatic effect, at MIC of 64.0 µg/mL (Crowle *et al*., 1988).

It has been hypothesized that PZA inhibits tubercle bacilli through its metabolite, pyrazinoic acid (POA). Tubercle bacilli are able to convert PZA to POA by the production of pyrazinamidase (Crowle *et al*., 1986). STR was found to be effective on macrophages at concentrations between 5.0 and 50.0 µg/mL (Crowle *et al*., 1984). EMB was found to inhibit the tubercle bacilli within the cells at the same concentrations as it did in bacteriologic culture medium. This occurs because macrophages enhance EMB effectiveness by killing tubercle bacilli, which have defective cell walls due to the effects of the drug (Crowle *et al*., 1985). RIF was found to be effective in macrophages at concentrations of 0.5 and 2.5 µg/mL (Duman *et al*., 2004).

8.3.3. Cytotoxicity activity

The cytotoxicity results indicated that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone demonstrated less toxicity, showing IC_{50} of 110.3 and 80.2 µg/mL respectively against the U937 cells. The ethanol extract of *G. africana* had an IC_{50} value at 120.0 µg/mL (Table 8.3).

The ratio of the IC_{50} values to MIC values of the ethanol extract, 2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone was less than 10, which indicates less activity and samples are not considered to be lead anti-TB samples. The significance of these values depends on factors such as components of the crude extract, compound structures and potential mechanism of action.

Table 8.3. Cytotoxicity of the ethanol extract and the isolated compounds against U937 cells

Plant species	MIC[a] (µg/mL)	IC_{50}[b] (µg/mL ± SD)	SI[c]
G. africana	1.20 x 10^3	120.0 ± 2.31	0.100
(2*S*)-5,7,2'-trihydroxyflavanone	100.0	110.3 ± 2.16	1.103
(*E*)-2',4'-dihydroxychalcone	50.0	080.2± 1.15	1.604
Doxorubin (positive drug)	nd[d]	1.153 ± 0.15	nd

[a]Minimum inhibitory concentration.

[b]IC_{50}: 50% inhibitory concentration of samples on U937 cell line.

[c]SI, selectivity index (*in vitro*): IC_{50} in U937 cells/MIC against *M. tuberculosis*. (If SI > 10, the compound is evaluated further).

[d]Not determined.

8.3.4. Subversive properties

In order to replicate and persist in its human host, *M. tuberculosis* must survive within the hostile environment of the macrophage, where bacterial oxidants, superoxide (O_2^-) and nitric oxide (NO^-) are generated in response to infection (Nathan and Shiloh, 2000). Two enzymes, NADPH oxidase and inducible nitric oxide synthase (NOS2), are largely responsible for production of O_2^- and NO respectively (Huang *et al*., 1993, Pollock *et al*., 1995). The O_2^- is reduced by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). A consequence of NADPH oxidative activity is that the phagocytosed bacteria are killed by oxidative damage to protein and DNA targets (Bhave *et al*., 2007). *M. tuberculosis* relies upon MSH for protection against toxic oxidants, for growth in an oxygen-rich environment and for establishing the pattern of resistance to TB drugs (INH and RIF). The metabolism of MSH involves Mtr (containing FAD as a cofactor), a member of the NADPH, which reduces FAD to the redox-active disulfide in Mtr (Bhave *et al*., 2007).

Our investigation on the NADPH oxidase activity of (2*S*)-5,7,2'-trihydroxyflavanone

with Mtr, found that this compound failed to exhibit any NADPH oxidase activity at 800.0 μM concentrations. Mtr is evidently not the target for the antitubercular activity of (2*S*)-5,7,2'-trihydroxyflavanone. The subversive substrate properties of naphthoquinones (**1-3, 5-6**, **12** and **22-24)** with Mtr and the comparisons of their MIC values (expressed as μM concentrations) in whole cell assays, are summarised in Table 8.4. Whilst K_m values show the substrate binding affinities and the kcat values express the maximum turnover rates, the overall catalytic efficiency (k_{cat}/K_m) best expresses the futile substrate efficiency of these substrates. It is the latter value that was used to look for a direct correlation between futile substrate properties (*in vitro*) and the whole cell antibacterial activities of these compounds. The K_m values of compounds **1**, **2**, **3**, **6**, **8** and **12** are all in the 200.0 – 500.0 μM range whereas **5**, **22** and **23** are significantly lower (30.0 – 60.0 μM). The previously reported K_m value of **2** with Mtr is 540.0 μM (Patel and Blanchard, 1999), which is comparable to the value determined herein. All of the naphthoquinones in Table 8.1 can be viewed as structural elaborations of the basic juglone scaffold **1** and in this context, it appears that methylation of the C-2 position or the 5-hydroxyl group are the most detrimental to substrate binding with Mtr.

Depending on which naphthoquinone motif acts as the electron acceptor during enzymatic turnover, diospyrin can be viewed as either a 2 - or a 6-substituted derivative of 7-methyljuglone. Similar observations can be made for neodiospyrin. These dimeric versions of 7-methyljuglone exhibit a 5 to 10-fold reduction in K_m. The turnover rates (k_{cat}) of the compounds in Table 8.4 are also reported. 7-methyljuglone and its 5-acetoxy derivative **8** have the fastest turnover rate at substrate saturation followed by **1** and **2**. The addition of the 8-chloro substituent (compounds **3**, **5** and **12**) is notably detrimental to the turnover rate at a level which is comparable to that observed for the naphthoquinone dimers **22** and **23**. In terms of substrate efficiency (k_{cat}/K_m) **5** is the most efficient subversive substrate with Mtr followed by **6** and **8**. There is no direct correlation between the antituberculosis activity in whole cell assays (MIC) and the k_{cat}/K_m values of these compounds with Mtr. Shinanolone lacks the conjugated benzoquinone motif that is required for subversive substrate activity

hence it is not a substrate for Mtr. It also displays significantly weaker antibacterial activity than any of the other naphthoquinones in whole cell assays as do compounds **17-19** which also lack the quinone motif (Weigenand *et al*., 2004). Comparing the Mtr substrate properties of the naphthoquinones (Table 8.4) with those observed for MSSM (Patel and Blanchard, 1999), it is evident that MSSM is turned over more efficiently. The absence of a direct correlation between the subversive substrate efficiency of these naphthoquinones and their MIC values is probably because their antituberculosis activity in whole cells is the accumulative consequence of their non-specific reactivity with multiple biological targets. In *M. tuberculosis* one of these targets could plausibly be Mtr. Additional targets could include other flavoprotein oxidoreductases such as lipoamide dehydrogenase and thioredoxin reductase, which unlike Mtr are also found in eukaryotes. It has also been suggested that naphthoquinone structures such as **6** could behave as non-functional ubiquinone and/or menaquinone surrogates, which may perturb electron transfer in respiratory chain processes (Patel and Blanchard, 2001).

Table 8.4. Substrate properties of substituted naphthoquinones with *M. tuberculosis* Mtr

Compound	K_m (μM)	k_{cat} s^{-1}	k_{cat}/K_m (x 10^{-5}s/μM)	MIC (μM)
1	324 (± 50)	1.088 (±0.079)	336	6
2	483 (±146)	0.732 (±0.140)	152	29
3	233 (± 45)	0.212 (±0.021)	91	45
5	36 (± 5)	0.592 (±0.017)	1644	22
6	254 (± 23)	2.365 (±0.214)	931	3
8	389 (± 59)	3.169 (±0.168)	815	11
12	435 (±112)	0.142 (±0.018)	33	85
22	33 (± 4)	0.118 (±0.005)	357	21
23	63 (± 8)	0.308 (±0.010)	488	27
24	----not active-up to 800 uM----			>530
MSSM	70[a]	6.667[b]	9524	----------
Isoniazid				0.2

[a]As reported in Patel and Blanchard, 1999

[b]Derived from the k_{cat} value (400/min) as reported in Patel and Blanchard, 1999

8.4. Conclusion

The resurgence of TB worldwide coupled with the increase in MDR *M. tuberculosis* has renewed an interest in the development of new antituberculosis treatment regimens (Cavalieri *et al*., 1995). A key target for antimycobacterial chemotherapy is cell wall biosynthesis. The complex lipoglycan calyx on the mycobacterial cell surface provides a significant physical barrier to intracellular-acting drugs. The lack of penetration is thought to be a reason why many antibiotics show no activity against *M. tuberculosis* (Gao *et al*., 2003). Since INH inhibits the biosynthesis of mycolic acids present in the cell wall of *M. tuberculosis,* this first-line drug is used in direct treatment programmes because of its activity against *M. tuberculosis*.

The identification of new antimycobaterial targets is essential to address MDR and latent TB infections. Numerous studies have validated amino acid biosynthetic pathways and downstream metabolites as antimicrobial targets and sulphur metabolic pathway are required for the expression of virulence in many pathogenic bacteria (Bhave *et al*., 2007). Any drug that could enhance the activity with other standard drugs like INH, EMB, PZA and STR would therefore be valuable in the treatment of TB.

Since (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone from *G. africana* showed synergistic activity with INH, it is speculated that the compounds might have similar mechanisms as that of INH. However, mechanistic studies on these compounds should be done in order to get an indication of 'flavonoids and chalcones' interference on mycolic acid synthesis, membrane synthesis and enzyme inhibition. The successful use of combinations of plant extracts is not only observed in anti-infective therapy, but also seen in the treatment of several disorders including cancer, HIV, inflammatory, stress-induced insomnia, osteoarthritis and hypertension.

The intracellular MIC of the ethanol extract of *G. africana* was higher than the extracellular MIC in this study. The activity might be due to *M. tuberculosis* being

unable to avoid macrophage killing and its survival during phagocytosis. This includes inhibition of phagosome-lysosome fusion, inhibition of the acidification of phagosomes, resistance to killing by reactive oxygen intermediates and modification of the lipid composition of the mycobacterial cell membrane, thereby altering its capacity to interact with immune or inflammatory cells. However, other mechanisms responsible for these properties should be identified, and this will represent potentially interesting targets for novel drugs and vaccines.

When a drug is used therapeutically, it is important to understand the margin of safety that exists between the dose needed for the desired effect and the dose that produces unwanted and possibly dangerous side effects (Labuschagne, 2008). The cytotoxicity results indicated that less toxicity of the ethanol extract of *G. africana*, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone against the U937 cells. This makes the ethanol extract of *G. africana* to be a candidate for herbal formulation and will have to be constituted for further investigation. Considering that a SI value > 10 is required for a compound to be selected for further testing, the results in this study indicated that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone are not antituberculosis leads due to their high MIC values.

In terms of substrate efficiency, the inactivity of (2*S*)-5,7,2'-trihydroxyflavanone is possibly due to its inactivity with Mtr which is found in *M. tuberculosis*. However, the mycothiol disulfide redox pathway is unique to actinomycetes (e.g. *M. tuberculosis*) and it substitutes the glutathione reductase pathway utilized in mammals. There is scope for narrowing the target specificity of (2*S*)-5,7,2'-trihydroxyflavanone and the naphthoquinones by appropriate structural modifications (e.g. carbohydrate/polyol motifs) so as to tailor their specificity for Mtr. Such an approach has previously been demonstrated by modification of simple naphthoquinone scaffolds with polyamine motifs to enhance their specificity for trypanothione reductase versus mammalian glutathione reductase and LADH enzymes (Salmon-Chemin *et al*., 2001). It will be interesting to investigate if the compounds have subversive properties with other enzymes.

CHAPTER 9
OVERALL DISCUSSION AND CONCLUSION

OVERALL DISCUSSION AND CONCLUSION

In South Africa, TB is the most commonly notified disease and the fifth largest cause of mortality, with one in ten cases of TB resistant to treatment in some areas. Many plants are used locally in traditional medicine to treat TB-related symptoms (McGaw *et al*., 2008). In this study, traditional healers and local people in areas of Venda region of Lipompo province, South Africa were interviewed, about medicinal plants species used traditionally to treat TB. The antimycobacterial activity tests of ethanol extracts and chemical constituents of selected medicinal plants (*A. afra*, *Dodonaea angustifolia, Drosera capensis*, *G. africana*, *P. africana*, *S. cordatum* and *Z. mucronata*), using different methods were investigated against selected two *Mycobacterium* species (*Mycobacterium smegmatis* and *M. tuberculosis*), which cause TB and infect the respiratory tract. Some of the selected plants showed antituberculosis activity *in vitro*. Ethanol extracts of *Dodonaea angustifolia, Drosera capensis* and *S. cordatum* showed inhibitory effects against *M. smegmatis*. The most potent plant was *G. africana* which showed goo d activity against both *Mycobacterium* species.

Due to the good inhibitory activity of the ethanol extract of *G. africana* amongst the other selected plants, the ethanol extract was selected for the isolation of compounds. Four compounds namely: (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-2',4'-dihydroxychalcone, (*E*)-3,2',4'-trihydroxychalcone and (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone, were isolated. Two compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone were previously isolated by other researchers and have been reported to have anticancer, antifungal, antibacterial and anti-inflammatory activity. Compound, (*E*)-3,2',4'-

trihydroxychalcone has not been reported from natural sources but previously isolated by Severi *et al*., 1998 and a novel compound, (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone is isolated from *G. africana* for the first time.

The compounds isolated were tested for antimycobacterial activity against *M. smegmatis* and *M. tuberculosis* using different methods. (2*S*)-5,7,2'-trihydroxyflavanone showed good inhibitory activity against *M. smegmatis* at 0.03 mg/mL. (*E*)-2',4'-dihydroxychalcone and the novel compound, (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone, both showed antimycobacterial activity against *M. tuberculosis* at 0.10 and 0.05 mg/mL respectively.

In terms of synergistic activity, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone reduced their original MICs four-fold resulting in an FIC of 0.5. The most pronounced effect of the two-drug action was demonstrated by both compounds with INH. The FIC index of (2*S*)-5,7,2'-trihydroxyflavanone and INH was 0.125 and for (*E*)-2',4'-dihydroxychalcone with INH was 0.1875 indicating synergistic activity.

The intracellular antimycobacterial activity of (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-2',4'-dihydroxychalcone and the ethanol extract of *G. africana* was good and MICs obtained were 50.0; 100.0 and 50.0 µg/mL respectively. This indicates that the ethanol extract could be more taken up by macrophages or other molecules in the cell, leading to the increased interaction with the bacteria.

The cytotoxicity results indicated that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone have less toxicity. During our investigation on the NADPH oxidase activity of only one compound, (2*S*)-5,7,2'-trihydroxyflavanone with Mtr, it was found that this compound failed to exhibit any NADPH oxidase activity at 800.0 µM concentrations. The mycothione reductase pathway is evidently not the target for the antitubercular activity of (2*S*)-5,7,2'-trihydroxyflavanone.

This is the first report of selected medicinal plants for their antimycobacterial activity

against *M. smegmatis* and *M. tuberculosis*. A good correlation between the susceptibility test results of the radiometric assay and microtitre dilution method was observed. Initial susceptibility testing of *M. smegmatis* is fast, economical and reproducible. The advantage of BACTEC radiometric assay is that it is rapid and the liquid medium used has more cell to drug contact and hence, the results are more accurate.

This study gives some scientific basis to the traditional use of these medicinal plants for TB as potential antimycobaterial agents and warrant further investigation. Many plant species are used traditionally in South Africa to alleviate symptoms of TB, and several interesting leads have originated for further inquiry following *in vitro* antimycobacterial activity evaluation. However, much work remains to be done on the systematic assessment of anti-TB efficacy of local plants against pathogenic *Mycobacterium* species, both *in vitro* and *in vivo*.

Finding new TB drugs represents a challenge and it is hoped that new compounds can be found that will reduce the treatment period. The methods of antimycobacterial screening and the way in which results are reported need to be standardized to enable comparison to be made between different researchers, as some authors report activity of extracts at 3.0 or 10.0 mg/mL while others including McGaw *et al* (2008), believe that only MIC values less than 0.1 mg/mL are worthy of labeling active. A screening program which was established by the National Institutes of Allergy and Infectious Diseases, screened over 50 000 compounds and an initial cutoff of 12.5 µg/mL was amended to 6.25 µg/mL to reduce the number of compounds to be screened further (Orme, 2001). From both the biological and the natural products chemical perspective, the various aspects of the collaborative challenge faced in TB drug discovery are applicable to other infectious agents.

In the present study it was found that the ethanol extract of *G. africana* showed moderate antituberculosis activity. However, the isolated compounds from the extract exhibited good MICs against *M. tuberculosis*. During synergistic studies, the findings were significant as combination of (2*S*)-5,7,2' trihydroxyflavanone and (*E*)-2',4'-

dihydroxychalcone reduced their original MICs four-fold. Hence the use of the combination of anti-TB compounds identified in this study / herbal extract together with conventional medicine can be recommended after evaluating the efficiency of the aforesaid extracts / compounds in the pre-clinical and clinical studies.

CHAPTER 10
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CHAPTER 11
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CHAPTER 12
APPENDICES - PUBLICATIONS

12.1 Publications resulting from this thesis:

Activity against *Mycobacterium smegmatis* and *M. tuberculosis* by extracts of South African medicinal plants. 2008. *Phytotherapy Research* **22**(6): 841 - 845

Activity of 7-methyljuglone derivatives against *Mycobacterium tuberculosis* and as subversive substrates for mycothiol disulfide reductase. 2007. *Biorganic Medicinal Chemistry* **15** (24): 7638 - 7646

12.2 Article submitted for review:

A new antimycobacterial chalcone isolated from *Galenia africana* L. var *africana*

Submitted March 2009: Journal of Natural Product

12.3 Chapter to be submitted in a book (In preparation)

Epidemiology, prevention and treatment of tuberculosis using medicinal plants and existing antituberculosis drugs

S.P.N Mativandlela and N. Lall

12.4 Conference presentation

12.4.1. 4th Advances in Synthesis and Chemical Biology Symposium, Dublin, UK. 5th December 2005

"Mechanistic studies of disulfide reductase inhibition by diospyrin, 7-methyljuglone and related anti-mycobacterial naphthoquinones isolated from *Euclea natalensis*'

S.P.N. Mativandlela, N. Lall, J.J.M. Meyer and C.J. Hamilton.

12.4.2. South African TB Conference, ICC Durban, South Africa. 1st - 4th July 2008

"Evaluation of flavonoid compounds alone and in combination against *Mycobacterium tuberculosis*"

S.P.N. Mativandlela, T. Muthivhi, M. van der Walt, N. Lall, J.J.M. Meyer and A.A. Hussein.

12.4.3. 39th Union World Conference on Lung Health. 16th – 20th October 2008. Palais des Congrès, Paris, France.

'Evaluation of selected South African medicinal plants against *Mycobacterium smegmatis* and *M. tuberculosis* for treatment of tuberculosis'.

S.P.N. Mativandlela, M. van der Walt, N. Lall, J.J.M. Meyer, T. Muthivhi