Pharmacological evaluation of South African medicinal plants used for treating tuberculosis and related symptoms

By

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Research Centre for Plant Growth and Development
School of Life Sciences
University of KwaZulu-Natal, Pietermaritzburg

November, 2014
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Signed at UKZN Pietermaritzburg campus on the day of November, 2014.

SIGNATURE………………………………………………
Declaration by Supervisors

We hereby declare that we acted as Supervisors for this PhD student:

Student’s Full Name: Balungile Madikizela

Student Number: 209523515

Thesis Title: Pharmacological evaluation of South African medicinal plants used for treating tuberculosis and related symptoms

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed Examiners.

__________________________
SUPERVISOR: PROFESSOR J. VAN STADEN

__________________________
CO-SUPERVISOR: PROFESSOR J.F. FINNIE
Publications from this Thesis


Details of Contribution to Publications that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1
Contributions: All experimental work and draft manuscript were performed by BM under guidance and supervision of ARN, JVS and JFF.

Publication 2
Contributions: All experimental work and draft manuscript were done by BM. ARN helped with experimental design. JVS and JFF supervised the study and edited the manuscript before submission.

Publication 3
Contributions: All experimental work and draft manuscript were done by BM. MAA assisted with the identification of compounds. JVS and JFF supervised and edited the manuscript before submission.

Author's abbreviation

<table>
<thead>
<tr>
<th>Author</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>Balungile Madikizela</td>
</tr>
<tr>
<td>ARN</td>
<td>Ashwell R. Ndhlala</td>
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<td>Mutalib A. Aderogba</td>
</tr>
<tr>
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<td>Jeffrey F. Finnie</td>
</tr>
<tr>
<td>JVS</td>
<td>Johannes Van Staden</td>
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</table>

Signed:........................................
Conference Contribution from this Thesis


Firstly, I thank God for his protection and for giving me a strong will and courage to pursue my studies.

The completion of this thesis was not an effort of my individual input but rather a collective effort of many people who contributed in various ways to help me to complete this work. Without their input, guidance, help, and encouragement it would not have been easy to accomplish this work.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-NQO</td>
<td>4-nitroquinoline-1-oxide</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATM</td>
<td>African traditional medicine</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus calmette-guérin</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicine</td>
</tr>
<tr>
<td>CFU/ml</td>
<td>Colony forming units/ml</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CPE</td>
<td>Cytopathic effects</td>
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<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOT</td>
<td>Directly observed treatment short course</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegration per minute</td>
</tr>
<tr>
<td>DPM background</td>
<td>Disintegration per minute background</td>
</tr>
<tr>
<td>DPM solvent blank</td>
<td>Disintegration per minute solvent blank</td>
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<tr>
<td>DPM extract</td>
<td>Disintegration per minute extract</td>
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<tr>
<td>DW</td>
<td>Dry weight</td>
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<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>Hydrochloric acid</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>ICC</td>
<td>Interim coordinating committee</td>
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<tr>
<td>INH</td>
<td>Isoniazid</td>
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<tr>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>Acronym</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>MDR tuberculosis</td>
<td>Multi drug-resistant tuberculosis</td>
</tr>
<tr>
<td>MECs</td>
<td>Members of executive council</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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<td>Mueller-Hinton</td>
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<td>MegaHertz</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>MRC</td>
<td>Medical Research Council</td>
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<td>MTT</td>
<td>Mitochondrial reduction</td>
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<tr>
<td>NEMA</td>
<td>National Management Act</td>
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<td>NMR</td>
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<td>National Research Foundation</td>
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<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>NTM</td>
<td>Non-tuberculous mycobacteria</td>
</tr>
<tr>
<td>NU</td>
<td>Natal University</td>
</tr>
<tr>
<td>OADC</td>
<td>Oleic acid, albumin, dextrose, and catalase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PG12</td>
<td>Prostaglandin 12</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
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<tr>
<td>REMA</td>
<td>Resazurin microplate assay</td>
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<td>TCM</td>
<td>Traditional Chinese medicine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>TLR-4</td>
<td>Toll-like receptor-4</td>
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<tr>
<td>TM</td>
<td>Traditional medicine</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydromethyl)aminomethane</td>
</tr>
<tr>
<td>UKZN</td>
<td>University of KwaZulu-Natal</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>XDR tuberculosis</td>
<td>Extensive drug-resistant tuberculosis</td>
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Abstract

Respiratory ailments are major human killers, especially in developing countries including South Africa. Tuberculosis is one of the most prevalent infectious respiratory tract disease posing a major threat to human healthcare worldwide. This disease is a highly contagious airborne bacterial disease that usually infects the lungs and sometimes other body parts. Tuberculosis spreads easily in overcrowded conditions from one person with an active respiratory disease to another via droplets that are emitted when they sneeze or cough. Approximately two million deaths that occur worldwide per annum are caused by tuberculosis and about 285,000 cases occur in South Africa. This is the seventh highest total number in the world. The emergence of drug-resistant tuberculosis and other pathogenic diseases over the past decades makes this disease a serious threat to human health worldwide. Emerging drug-resistant tuberculosis strains and the long duration of treatment has established an urgent need to search for new effective agents. According to a 2012 report by the World Health Organisation (WHO), South Africa, China, India and Russia are the countries with the highest prevalence of multi drug-resistant (MDR) tuberculosis.

Most researchers in South Africa have focused on evaluating the antimycobacterial activity of medicinal plants against bacterial strains that cause tuberculosis, but there has not been sufficient focus on the related ailments. Therefore, one of the aims of the present study was the evaluation of the antimicrobial properties of the selected medicinal plants against Mycobacterium species and other bacterial strains related to respiratory infection. The floral diversity of South Africa has a potential for yielding new bioactive compounds, therefore pharmacological screening of plant extracts from this region is important. The aim of this study was the pharmacological evaluation of plants that are used traditionally in South Africa to treat tuberculosis and related symptoms against microorganisms that cause respiratory ailments, and the identification of compounds from antimicrobial active plant extracts.

Ten plants: Abrus precatorius subsp. africanus (leaves and seeds), Asparagus africanus (leaves), Asparagus falcatus (leaves), Bransviglia grandiflora (bulb), Ficus sur (bark and roots), Indigofera arrecta (leaves and roots), Leonotis intermedia (leaves and stem), Pentanisia prunelloides (leaves and roots), Polygala fruticosa (whole plant), and Terminalia phanerophlebia (leaves, roots and twigs) were selected based on a survey of available
literature of medicinal plants used in South Africa for the treatment of tuberculosis and related symptoms. Ground plant material from different plant parts of the 10 plants were extracted sequentially with four solvents: petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) as well as water, and a total of 68 extracts were produced. The plant extracts of the selected plants were evaluated for antibacterial activity against four microorganisms (Klebsiella pneumoniae, Staphylococcus aureus, Mycobacterium aurum A+ and Mycobacterium tuberculosis H37Ra) associated with respiratory infections using the microdilution assay. Cyclooxygenase-2 (COX-2) enzyme was used to evaluate the anti-inflammatory activity of the extracts. The Ames test and mitochondrial reduction (MTT) assays were used to establish toxicity of the extracts that showed noteworthy antimicrobial activity against the tested bacterial strains (Klebsiella pneumoniae, Staphylococcus aureus, Mycobacterium aurum A+ and Mycobacterium tuberculosis H37Ra). The extracts were tested for genotoxicity against Salmonella typhimurium (TA98 and TA100 strains) and cytotoxicity using monkey kidney Vero cells. Based on good antimicrobial activity observed, compounds were isolated from Terminalia phanerophlebia (leaves). Crude extracts obtained from 80% methanol (MeOH) of Terminalia phanerophlebia were successively extracted with hexane, DCM, ethyl acetate (EtOAc) and n-butanol. The fractions and isolated compounds obtained were tested for their antibacterial activity against Mycobacterium aurum A+, Mycobacterium tuberculosis H37Ra, Staphylococcus aureus and Klebsiella pneumoniae. Structure elucidation was carried out using NMR (1D and 2D) spectroscopic methods.

This investigation revealed the pharmacological potential of the 10 plants used in South Africa for traditional treatment of tuberculosis and related symptoms: Abrus precatorius subsp. africanus (leaves and seeds), Asparagus africanus (leaves), Asparagus falcatus (leaves), Brunsvigia grandiflora (bulb), Ficus sur (bark and roots), Indigofera arrecta (leaves and roots), Leonotis intermedia (leaves and stem), Pentanisia prunelloides (leaves and roots), Polygala fruticosa (whole plant), and Terminalia phanerophlebia (leaves, roots and twigs). The minimum inhibitory concentration (MIC) values of the tested plant extracts ranged from 0.098 to 12.5 mg/ml. Out of 68 extracts tested from different plant parts of the 10 plant species, 18 showed good antimicrobial activity against at least one or more of the microbial strains tested with MIC values ranging from 0.098 to 0.78 mg/ml. For anti-inflammatory results, only three extracts showed high inhibition (> 70%) of the COX-2 enzyme. In the Ames test using Salmonella typhimurium (TA98 and TA100 tester strains), all the extracts tested were non-genotoxic. However, in the MTT assay nine extracts demonstrated
cytotoxicity. Bioguided fractionation of the EtOAc fraction of *Terminalia phanerophlebia* (leaves) afforded two bioactive compounds: methyl gallate (methyl-3,4,5-trihydroxybenzoate) (1) and a phenylpropanoid glucoside; 1,6-di-O-coumaroyl glucopyranoside (2). These compounds are reported from *Terminalia phanerophlebia* for the first time. Both compounds showed good antimicrobial activity against all bacterial strains tested with MIC values ranging from 0.063 to 0.25 mg/ml. Inhibition of *Mycobacterium tuberculosis* by 1,6-di-O-coumaroyl glucopyranoside (2) at a MIC value of 0.063 mg/ml was noteworthy, as this bacterial strain is reported to be the leading cause of tuberculosis worldwide.

The good antimicrobial property of *Abrus precatorius* subsp. *africanus*, *Asparagus africanus*, *Asparagus falcatus*, *Terminalia phanerophlebia*, *Indigofera arrecta*, *Ficus sur*, *Leonotis intermedia* and *Pentanisia prunelloides* partially authenticate their traditional use in the treatment of respiratory diseases. However, these plants must be used with caution as some of their extracts showed cytotoxicity against Vero cells. The results observed in this study indicate that *Abrus precatorius* subsp. *africanus*, *Asparagus africanus*, *Asparagus falcatus*, *Ficus sur*, *Pentanisia prunelloides* and *Terminalia phanerophlebia* could be investigated further against drug-resistant tuberculosis strains. Good antimicrobial activity exhibited by the compounds isolated from *Terminalia phanerophlebia* (leaves) authenticate the traditional use of this plant in treating tuberculosis and its related symptoms. Compound (2), 1,6-di-O-coumaroyl glucopyranoside showed noteworthy activity against a *Mycobacterium tuberculosis* strain H37Ra (0.063 mg/ml), therefore this compound could potentially serve as a lead in tuberculosis drug discovery.
Chapter 1: Introduction and literature review

1.1. Introduction

Over the centuries, humans have depended on different parts of plants for basic needs such as food, clothing, and shelter (PHILLIPSON, 2001). Plants have been used as medicine since antiquity to cure various human diseases and ailments, they have also been used for other purposes such as production of fertilisers, flavours, and fragrances (HALBERSTEIN, 2005; GURIB-FAKIM, 2011). Traditional medicine systems like the Chinese, Ayurvedic, Native American, and African ones have been in existence for centuries and they continue to provide mankind with new remedies (SALIM et al., 2008). This is evident from archaeological proof indicating that people regularly used medicinal plants in ancient times for curative and psychotherapeutic purposes (HALBERSTEIN, 2005). According to NEWMAN et al. (2000) “the first annals on the therapeutic properties of medicinal plants dates back to 2600 BC”. The annals were written on clay tablets in cuneiform, from Mesopotamia (DIAS et al., 2012). The oils of Cedrus species and Cupressus sempervirens L., Glycyrrhiza glabra L., Commiphora species and Papaver somniferum L. are among the substances that were used, all of which still continue to be used today for the treatment of coughs, colds, parasitic infections and inflammation (GURIB-FAKIM, 2006). In developing countries people still use medicinal plants as their primary source of medicine and approximately 60-80% of the world’s population use plants for their healthcare needs (PALOMBO, 2006). Although there has been universal, cross-cultural utilisation of medicinal plants, scientific research validating their efficacy is being conducted on a worldwide scale (KONG et al., 2003). There are many research journals available, highlighting the biological activities and constituents of medicinal plants. Some of the therapeutic properties accredited to plants have been proven to be noteworthy, whereas others erroneous, yet the interest in plants as a source of potential chemotherapeutic agents continues (GURIB-FAKIM, 2011).

1.2. History of the medicinal use of plants by humans

The commencement of the use of medicinal plants by humans was instinctive, based on experience, as there was no information concerning which plant to use and how it could be used (PETROVSKA, 2012). As time passed, reasons for specific medicinal plant usage in
treating certain illnesses became available; therefore, the use of plants as medicine gradually abandoned the empiric basis and became founded on descriptive facts. Plants were used as the source of treatment and prophylaxis until the advent of chemical medicine in the 16th century. However, the decreasing effectiveness of synthetic drugs make the use of natural remedies interesting once more.

According to CRABTREE (2013), the Sumerian clay slab from Nagpur (about 5000 years old) contained written proof of the use of plants for preparation of drugs. It contained 12 methods for drug preparation referring to over 250 plants. Camphor, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra are some of 365 dried plant parts recorded in ‘Pen T’Sao’, a Chinese book on roots and grasses written by Emperor Shen Nung approximately 2500 BC (BENDER et al., 1966). Spices used today such as nutmeg, pepper, and clove originate from India, and are recorded in Indian holy books ‘Vedas’, as used for treatment of illnesses. A collection of 800 prescriptions referring to 700 plant species and drugs used for therapy such as pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander, juniper, and common centaury are recorded on the Ebers Papyrus written approximately 1550 BC (BENDER et al., 1966).

According to information from the Bible and the holy Jewish book (The Talmud), aromatic plants such as myrtle and incense were used by humans during various rituals accompanying a treatment (PETROVSKA, 2012). Medicinal plants such as wormwood and common centaury were applied against fever; and garlic against intestinal parasites. Opium, henbane, deadly nightshade, and mandrake were used as narcotics; fragrant hellebore and haselwort as emetics. Sea onion, celery, parsley and asparagus were found in the works of the Hippocrates, classifying plants based on their physiological action (PETROVSKA, 2012).

Theophrastus was considered the founder of botanical science with his books “De Causis Plantarium”- Plant Etiology and “De Historia Plantarium”-Plant History (THANOS, 2005). He produced a classification of more than 500 medicinal plants known at the time and amongst those plants there were cinnamon, iris rhizome, false hellebore, mint, pomegranate, cardamom, fragrant hellebore and monkshood (THANOS, 2005).

In early history, Dioscorides was the most prominent writer on plant drugs and is today known as the father of pharmacognosy, who studied medicinal plants wherever he travelled
while working as Nero's army military physician and pharmacognosist (PETROVSKA, 2012). Paracelsus was one of the advocates of chemically prepared drugs from plants and minerals. His beliefs were based on observations, and he simultaneously supported the “Signatura doctrinae”-signature doctrine (PETROVSKA, 2012).

Although the early use of medicinal plants by ancient people was as simple pharmaceutical forms, infusions, decoctions and macerations, between the 16th and 18th centuries, the demand for compound drugs was increasing. The compound drugs contained medicinal plants along with drugs of an animal and/or plant source. The early 19th century saw the turning point in the use and knowledge of medicinal plants as there was the discovery, substantiation and isolation of alkaloids, glycosides, tannins, saponosides, etheric oils, vitamins and hormones (ZENK and JUENGER, 2007). Today, almost all pharmacopoeias worldwide describe plant drugs of medicinal value.

1.3. An overview of traditional medicine systems

Traditional medicine (TM), variously known as ethnomedicine, folk medicine, native healing, or complementary and alternative medicine (CAM) is an ancient and culture-bound method of healing that humans have used to cope and deal with various diseases that have threatened their existence and survival (ABDULLAHI, 2011). Several classifications have been attempted for defining and classifying TM (WACHTEL- GALOR and BENZIE, 2011). However, the World Health Organisation (WHO) defined TM as the “sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness” (WHO, 2000).

Traditional medicine is used by a large proportion of the population either due to the system being accepted from a cultural and spiritual perspective or to the high cost of western pharmaceuticals (TAYLOR et al., 2001). There are many forms of TM systems that exist within cultures and in different regions (WACHTEL-GALOR and BENZIE, 2011). Regarding raw material, production methods and quality control of products, in South Africa
and many other developing countries, there is currently no standardisation of TM (TAYLOR et al., 2001).

African and Latin American TM is marked strongly by an oral tradition of sharing information about medicinal plants even though training exists in various forms (from self-education to teaching by an expert practitioner) (UNESCO, 2013). On the other hand traditional Chinese medicine (TCM) has a formal structure and documentation is supported by the government (TAYLOR et al., 2001).

Traditional Chinese medicine is an ancient system (more than 3000 years) of medicine that is based on two separate theories about the natural laws that govern good health and longevity, namely ‘yin’ and ‘yang’, and the five elements (‘wu xing’) (WACHTEL-GALOR and BENZIE, 2011; YAO et al., 2013). Traditional Chinese medicine was incorporated into the national healthcare system by providing academic training and emphasising research and today it is taught in local medical schools and universities as part of a mixed curriculum (GURIB-FAKIM, 2006). In TCM, 60% of the curriculum is devoted to TM and the remaining 40% to modern medicine (WACHTEL-GALOR and BENZIE, 2011). Up to 40% of healthcare delivered in China is TCM and more than 90% of general hospitals have TM units (WACHTEL-GALOR and BENZIE, 2011).

Among the systems of medicine, African traditional medicine (ATM) is perhaps the most diverse. However, in the past the knowledge was usually transferred orally from one generation to the next, thus it is poorly recorded when compared to the TCM (MAHOMOODALLY, 2013). African traditional medicine in its diverse forms, is holistic involving both the body and the mind, with the healer typically diagnosing and treating the psychological basis of an illness before prescribing medicines to treat the symptoms (GURIB-FAKIM, 2006). The majority of the African population (80%) use TM for their primary healthcare needs (RICHTER, 2003). Some people in Africa utilise only TM, while others combine it with conventional drugs. Traditional medical practitioners (TMPs) are an important aspect of the health-care delivery system (ADEDEJI et al., 2013).

Africa is known to have a rich biological and cultural diversity with marked regional difference in healing practices, and is considered as the cradle of mankind (DA SILVA et al., 2011). There is an estimated 68000 plant species that are found in the African region of
which about 3500 are known to be endemic (ADEDEJI et al., 2013). More than 5000 plants are known to be used for curing various diseases and ailments, but only a few have been studied extensively (IWU, 1993).

In the recent past, there has been a growing interest in TM and its relevance to public health both in developed and developing countries. There is also a critical need to mainstream TM into public healthcare to achieve the objective of improved access to healthcare facilities (UNESCO, 2013). Traditional medicine practice is also spreading significantly in some developed countries as people become disillusioned by modern medicine and lose confidence in it. Almost half the population in industrialised countries use TM, either because they are convinced that this type of medicine is risk free and more natural (UNESCO, 2013). Although many developing and developed countries recognise TM, not all of them make it an important part of the healthcare system. However, a few countries such as China and India have reached a level of offering TM as a healthcare option.

Due to the fact that TM lacks a scientific basis, although contributing significantly in healthcare it seldom cooperates with modern healthcare (TAYLOR et al., 2001). Other problems associated with TM are that its methods, and training are often kept secret, and does not keep pace with advances in science and technology (TAYLOR et al., 2001). The developed world initially showed rather little attention to indigenous traditional knowledge and provided minimal help to developing regions for preservation, collection and systemisation of the knowledge until the late 1980s (TAYLOR et al., 2001). However, recently there is an interest in natural products, this has led to an increasing respect for indigenous people and their customs and beliefs.

1.4. South Africa’s traditional plant based medicines

South Africa is estimated to have more than 24 000 plant species that occur within her borders, representing 10% of the world’s species. Thus, the South African region is considered to be a biodiversity hot spot (COETZEE et al., 1999). About 3000 plant species have been recorded by various cultural groups in South Africa as part of their materia medica (DE WET et al., 2013). Traditional medicine still forms an important part of the healthcare system of the South African population as is the case in most developing countries. It was
estimated that up to 60% of the South African population consult a traditional healer in addition to western trained doctors (TAYLOR et al., 2001). The South African population does not only use plants as medicine due to the rich biodiversity that exist in this region, but also because of coexisting diverse cultural groups with their associated beliefs and practices (PRETORIUS, 1999). Each culture in this region uses medicinal plants for preventing and curing various diseases and ailments. Traditional medicine treatment used in South Africa is holistic, it does not only deal with the physical aspect of the disease but also deals with psychosocial aspects (PRETORIUS, 1999).

A traditional healer is defined as a competent and community recognised person who provides healthcare using natural resources and has relevant knowledge about attitudes and beliefs regarding physical, mental and social well-being and the causation of disease (PRETORIUS, 1999; ELUJOBA et al., 2005). Although diverse cultural groups within South Africa subscribe to their own traditional systems of therapy, there are structural similarities that are evident. With different African cultures TM practitioners are classified into different categories as they do not perform the same functions. Among practitioners of TM are the diviner, they are known by different names in the different cultures of South Africa, for example, amagqirha in Xhosa, izangoma in Zulu, ngaka in Northern Sotho, selaoli in Southern Sotho and mungome in Venda and Tsonga (PRETORIUS, 1999; TRUTER, 2007). The popular press terms the diviner as isangoma, and the herbalist inyanga. However, nowadays the distinction between the diviner and the herbalist is not clear as a result of overlapping duties amongst the two. The prophet or faith healer that divines and heals within the framework of the African independent church is a more recent category of traditional healers. The interim coordinating committee (ICC) of traditional medical practitioners in South Africa has proposed to include traditional surgeons (iingcibi in Xhosa), and traditional midwives/birth attendants (ababelethisi in Zulu) in the proposed legislation. The healer: population ratio in South Africa is estimated to be 1:200. There are approximately 200 000 traditional healers in South Africa (TAYLOR et al., 2001; TRUTER, 2007). However, this is likely to be an underestimation of people who provide medicinal plants-dependent services, as there are chances of ignoring people who do not identify themselves as full-time traditional healers, although they use medicinal plants to provide healthcare. Traditional healers are unlike medical doctors who only treat the disease, but also try to relink the social and emotional equilibrium of patients based on community rules and relationships. In many communities, traditional healers usually determine how to bring the sick person back into
harmony with the ancestors by acting as intercessors amongst the visible and invisible worlds; the living and the dead or ancestors, determining which spirits are working (ABDULLAHI, 2011). However, a significant turning point in the history of this old tradition and culture was marked by the influx of Europeans. Western medicine and culture introduction gave rise to a cultural-ideological clash which had previously created an unequal power relation that undermined and stigmatised the indigenous African way of medicine that included the use of medicinal plants (ABDULLAHI, 2011). However, that is no longer the case as medicinal plants are known to have great potential in human healthcare.

The majority of South Africans first call traditional healers when illness strikes (TRUTER, 2007; VAN NIEKERK, 2012). The reason for calling traditional healers first might be because they are easily accessible as they reside within the communities and that people believe in their healing abilities or in alleviating illnesses. Traditional healers are involved in activities such as tuberculosis control, nutritional, and social plant use programmes, and are part of student training programmes, thus playing a significant role in dealing with health problems in South Africa. Additionally, the acquired immunodeficiency syndrome (AIDS) foundation of South Africa, which supports human immunodeficiency virus (HIV) and AIDS education for traditional healers recognises that they are engaged in educating people and providing counselling (VAN NIEKERK, 2012).

Due to the fact that traditional healing techniques vary among geographic regions and cultures, the methods of healing need to be standardised. Standardisation of TM in South Africa has recently become a topic of discussion based on the fact that there is loss of oral information and there is a rapid pace of urbanisation in this country (TAYLOR et al., 2001). National governments have made moves to encourage research into medicinal plants and the development of good policies, regulations and standards (trade or use of medicinal plants and training of traditional healers) by setting up research institutes and funding. In 1995, the South African government took the initiative for making ATM legitimate. The provincial governments were called by the national health minister and the provincial Members of Executive Council (MECs) for health to conduct public hearings on traditional health care availability (PRETORIUS, 1999). In 2011, a call was made by the president of the Republic of South Africa for standardisation of ATM. A follow up conference was held in South Africa in 2013 to determine how other countries such as China and Ghana standardised their traditional medicines and to use that as a guide to standardise the ATM in this region. Greater
interest in research of South Africa’s natural resources is being promoted by the country’s government through agencies such as the National Research Foundation (NRF) and Medical Research Council (MRC).

1.5. Secondary metabolites

A plant is similar to a factory, as it produces a vast and diverse range of organic compounds classified as primary and secondary metabolites (CROZIER et al., 2006). Primary metabolites are defined as compounds that have essential roles in processes such as photosynthesis, respiration and plant growth and development (CROTEAU et al., 2000; CROZIER et al., 2006). Examples of primary metabolites include phytosterols, acyl lipids, nucleotides, amino acids and organic acids. On the other hand, secondary metabolites are structurally diverse compounds and vast numbers are distributed among plant species. Examples of secondary metabolites include terpenes, phenolic compounds, and alkaloids, classified based on their biosynthetic origin (AGOSTINA-COSTA et al., 2012). Although ignored for some time, their role in plants is now attracting attention as some of these compounds have key roles in plant protection from microbial infection. Secondary metabolites are also of interest as they are viewed as potential sources of new natural drugs, antibiotics, and insecticides (CROZIER et al., 2006). A vast number of plant based natural products have been reported to provide a beneficial role in human health. This has inspired more research focusing on searching for new drugs and antibiotics (CROTEAU et al., 2000).

1.6. Drug discovery from medicinal plants

Natural products with therapeutic properties have been utilised as the main source of drugs for a long time (RATES, 2001). Between 1981 and 2002, about 28% of new chemical entities were natural product derivatives (BALUNAS and KINGHORN, 2005). Various infectious diseases and ailments are known to be treated by the use of herbal remedies. Presently natural products and their derivatives represent more than 50% of all drugs in clinical use and 25% of that, originate from higher plants (MARIDASS and DE BRITTO, 2008). Out of 252 drugs considered by the WHO as basic and important, 25% are exclusively of plant origin (RATES, 2001). Examples of some 100 plant-based drugs that were introduced in United States of America (USA) drug market during 1950-1970 include
deserpidine, reseinnamine, reserpine, vinblastine and vincristine which are derived from higher plants. Other plant derived drugs such as artemisinin appeared worldwide from 1971-1990 (VERMA and SINGH, 2008). Early drugs such as cocaine, codeine, digitoxin, morphine and quinine, some of which are still in use, were derived from medicinal plants (BALUNAS and KINGHORN, 2005).

In developing countries, about 33% of drugs that were produced in the early 1970s were derived from plants. This accounted for up to 40% of all prescriptions at that time (KONG et al., 2003). In 1980, more than US $1800 million was paid by consumers to the American economy in prescriptions containing plant-derived drugs (FARNSWORTH et al., 1985). In 1985 the annual production of traditional plant remedies contributed US $571 million dollars to the Chinese economy and sales of crude drugs in the country amounted to $1400 million (AKERELE, 1993). This shows the importance of plant-derived drugs in boosting the economy of developed and developing countries.

Ethnobotany and plants provide a rich resource of natural drugs for scientific development. However, a very small percentage (about 15%) of plants have been investigated for their potential as sources of drugs and only about 5% for one or more biological activities (KONG et al., 2003; VERPOORTE et al., 2006). Even though there is a lot of research on medicinal plants that is published yearly, there are only a few plants that have been studied broadly for pharmacological properties (KONG et al., 2003). Due to these facts, medicinal plants obviously represent a great source of future new leads for the development of drugs.

Drugs discovered from plants benefit humans even today, although earlier, it was mostly a result of coincidence based on human practices (WANG et al., 2007). The isolation and characterisation of pharmacologically active compounds from medicinal plants continues and recently, drug discovery techniques have been applied to the standardisation of herbal medicines, to elucidate analytical marker compounds (BALUNAS and KINGHORN, 2005). Natural products, particularly medicinal plants continue to be important sources of new drugs in spite of the current interest in molecular modelling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies (DIAS et al., 2012). These compounds can be used as leads for new drugs and new chemical entities other than being used directly as drugs (SALIM et al., 2008). However, in some situations these compounds
are not the first choice in treatment of severe life-threatening infections, but can be useful in many other ways (VAN WYK and WINK, 2004).

1.7. Respiratory tract infections

The respiratory system is made up of two parts classified as the upper and lower respiratory tract (FILE, 2002). Infectious diseases of the respiratory tract are common and they present a challenge in precise diagnosis and management, and are among the leading causes of mortality and morbidity worldwide (ELLIS, 1998; FILE, 2000). These infections occur among both the young and elderly with aged humans having a greater mortality from pneumonia, influenza, tuberculosis, infective endocarditis, urinary tract and intraabdominal infections (FILE, 2002). Depending on the part of the respiratory tract that is affected, infections can be caused by a huge variety of microorganisms. Most upper respiratory tract infections are caused by viral pathogens, they are not severe, and are self-limiting. In contrast, the lower respiratory tract infections are more likely to have a bacterial etiology, are more severe, and more often require antimicrobial intervention (FILE, 2002). Annually, about 7 million people die worldwide as a direct consequence of acute and chronic respiratory infections from the common cold to many life-threatening conditions such as tuberculosis (AKINGBADE et al., 2012). Infectious diseases of the respiratory tract are a widespread problem and will likely remain a problem for the future (ELLIS, 1998).

Globally, lower respiratory tract infections are a common reason for hospital outpatient visits and antimicrobial prescriptions (FILE, 2002). In 1998, the WHO reported that 3.7 million deaths were due to lower respiratory tract infections (CARROLL, 2002). Despite all the advances made by medical science, lower respiratory tract infections are still the most frequent among infectious causes of mortality worldwide (AKINGBADE et al., 2012). Pulmonary tuberculosis is one of respiratory tract infections that arise from pathogens in the lower respiratory tract. According to WHO, tuberculosis is one of the most prevalent causes of death in Africa, responsible for about 1.5 million deaths in 2008 (BATES et al., 2013).

1.7.1. Overview of tuberculosis
Tuberculosis is an airborne bacterial disease that is highly contagious mostly infecting the lungs and sometimes other body parts like the central nervous system, adnexa, prostate seminal vesicles, bladder, ureter, adrenal glands, spleen, liver, peritoneum, bones, spine, psoas muscles, intestine, tonsils and middle ear (OKUNADE et al., 2004). The disease can be classified as pulmonary or extrapulmonary tuberculosis, and is usually caused by a human tubercle bacillus called *Mycobacterium tuberculosis* but occasionally by the bovine tubercle bacillus, *Mycobacterium bovis* or by *Mycobacterium africanum* (TRIPATHI et al., 2004). Tuberculosis is one of the most prevalent infections of mankind having a major challenge in public health contributing considerably to illness and death worldwide (AHMAD, 2011). In the case of pulmonary tuberculosis, the bacterium usually destroys the soft tissue of the lungs, resulting in difficulty with breathing, and this cause cavities or holes in the lungs, and blood can be coughed up. If untreated it can cause death. The bacterium is very resistant to sunlight, antiseptics and disinfectants and can survive in dry sputum for weeks. Tuberculosis spreads easily in overcrowded conditions from one person to another via droplets from the throat and lungs of people with the active respiratory disease when they sneeze or cough (GREEN et al., 2010; TEKWU et al., 2012). Transmission of tuberculosis occurs after prolonged close contact with a person that tested smear positive for the bacteria. The symptoms of tuberculosis vary depending on the part of the body that is infected. However, the most common tuberculosis symptoms may include persistent cough (for three weeks or more), chest pains, tiredness and weakness of the body, loss of appetite and weight, night sweats even when it’s cold and coughing up blood or mucus, fever, easy fatigability, stiffness of the neck, headache, vomiting and skin rash (MOORE et al., 2009). Tuberculosis infection can be latent (showing no symptoms of tuberculosis infection while the bacteria remains alive and dormant) or active (have symptoms of tuberculosis infection). Tuberculosis infection is latent at the initial stage of infection and may progress to an active disease in later life particularly if a person’s immune system becomes weakened, for example in the case of HIV/AIDS or due to chemotherapy. The majority of tuberculosis infected people are unaware of their infection as they appear not to be sick. However, when the disease starts to be active they may die if they do not receive effective treatment immediately.

### 1.7.2. Historical viewpoint of tuberculosis

Historically, tuberculosis is one of the oldest, most prevalent and diverse health problems of mankind known since time immemorial (AHMAD, 2011; FYHRQUIST et al., 2014).
Scientists believe that the disease established itself in human populations about 8000 B.C. at the time of transition of humans from nomadic tribes to settled, agricultural based societies (DANIEL, 2006). Tuberculosis has been detected in prehistoric skeletons and Egyptian mummies, and reference was found in ancient Babylonian and Chinese writings (PAVAN et al., 2012). Many of the human remains found in Egypt show vertebrae erosion and fusion due to infection by tuberculosis. By the time the Europeans reached East Africa in the 19th century tuberculosis was already well established (DANIEL, 2006). During the emergence of Europe from the dark ages, a renewed interest in science coincided with an tuberculosis epidemiology that began in early 1600 and continued for the next century (BASEL, 1998). Tuberculosis became known as the white death and was almost as feared as the black death also known as bubonic plague. In America, just like in Egypt, there is archeological evidence of early tuberculosis in Peruvian mummies. There is evidence that tuberculosis in America occurred prior to the arrival of the first European explorers (DANIEL, 2006). In ancient Greece, tuberculosis was called phthisis (consumption) and was noted by a Greek physician, Hippocrates (BASEL, 1998; DANIEL, 2006). In the beginning of the 20th century despite the rapid spread of tuberculosis in Europe, Nilotic North Africa, and America, in sub-Saharan Africa tuberculosis remained unknown, as has been reported by a number of medical observers (BLOOM, 1994). As noted by the army medical officers from Britain in places where immigration of Europeans had not occurred, tuberculosis was unknown. Livingstone and Lichtenstein also found no trace of tuberculosis in parts of South Africa in the first half of the 20th century (BLOOM, 1994).

In the Biblical books of Deuteronomy and Leviticus, tuberculosis is noted clearly using the ancient Hebrew word schacheopheth (DANIEL, 2006). René Théophile Hyacinthe Laennec clearly identified the pathogenesis, described the physical signs of tuberculosis and also unified its concept whether pulmonary or nonpulmonary. By the time of Laennec, tuberculosis had surged across Europe, with London, Stockholm and Hamburg having a death rate approaching 800-1000 per 100000 annually (DANIEL, 2006). American cities also had similar death rates. During such tuberculosis incidences, the society responded by romanticising the disease with victims wan and pallid faces thought to be attractive (BASEL, 1998). Hermann Heinrich Robert Koch changed the history of tuberculosis when he produced evidence that the disease was caused by a specific microbe. Using a staining technique he visualised slender rods which he called tubercle bacilli. Wilhelm Conrad von Röntgen also
contributed to the diagnosis of tuberculosis by discovering X-rays that showed the development, course and severity of the disease (BASEL, 1998).

In the mid-19th century the mortality rate of tuberculosis began to decrease due to advanced knowledge about the disease and the use of vaccines and several antibiotics to control it (DANIEL, 2006). The decline of tuberculosis is still evident in Europe and America with the incident rate continuing to be low (DANIEL, 2006; WHO, 2013). However, Africa remains the only continent where tuberculosis rates are still increasing (KAMATOU et al., 2007).

1.7.3. *Mycobacterium*

Mycobacteria, belonging to the family Mycobacteriaceae are made up of Gram-positive, aerobic, non-motile and slightly curved or straight rod shaped bacteria that are characterised by a complex cell envelope containing a high percentage of lipids which includes mycolic acid (PARISH and STOKER, 1998; ROGALL et al., 1990; DOSTAL et al., 2003; HASAN et al., 2013). The cell envelope isolates the cell from the environment and protects it from adverse conditions as well as making the bacteria relatively impermeable to antibiotics (PARISH and STOKER, 1998). Mycobacteria appear phenotypically most closely related to *Nocardia*, *Rhodococcus* and *Corynebacterium* (DOSTAL et al., 2003). It can infect most animal species, however most interest resides in the fact that it includes major human pathogens like *Mycobacterium tuberculosis* (the most disease infectious cause of mortality worldwide) and *Mycobacterium leprae* (PARISH and STOKER, 1998). Naturally and taxonomically mycobacteria falls into two major groups: the slow growers and fast growers, the former includes most of the human and animal pathogens whereas the latter consist of non-pathogenic species such as *Mycobacterium aurum*. The pathogenic species of mycobacteria that cause tuberculosis in humans and animals include *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium canetti*, *Mycobacterium africanum*, and *Mycobacterium microti* (McGAW et al., 2008a). The non-tuberculous group of mycobacteria that are mostly wide spread in the environment include *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium paratuberculosis* and *Mycobacterium lepraemurium*. The nonpathogenic strains of mycobacteria are encountered after surgery and as opportunistic infections in immunocompetent and immunocompromised individuals.
1.7.4. Global effect of tuberculosis

Tuberculosis has reemerged in recent years as a serious world health problem (CAMACHO-CORONA et al., 2007; MANN et al., 2008; McGAW et al., 2008a; SHUBLADZE et al., 2013). It is the most common cause of morbidity and mortality; more especially in HIV infections, and is becoming pandemic in some parts of the world (GANDHI et al., 2006). There is estimation that one-third of the world’s population is infected with the *tubercle bacillus* (KAMATOU et al., 2007; BANSAL et al., 2009; BUENO et al., 2011; JENA et al., 2013). Approximately eight million new cases and two million deaths per annum are caused by tuberculosis (BUENO et al., 2011). In 2008, South-East Asia was declared by the WHO as a region having the largest number of new tuberculosis cases with 35% of newly reported cases globally. The sub-Saharan Africa had over 350 cases per 100000 members of the population killing both adults and children (WHO, 2010; RAMOS et al., 2013). Africa also has the worse death rate of tuberculosis patients. This is due to failing tuberculosis programs (GANDHI et al., 2006; MAKOMBE, 2006). Out of an estimated 1.7 million people that died from tuberculosis in 2009, the African region had the highest number (WHO, 2010). Tuberculosis remains a world health problem (WHO, 2009a; WHO, 2011).

Africa which is home to approximately 11% of the world’s population reports more than a quarter of the global tuberculosis burden and the spread of this disease is still rising in the continent (MAKOMBE, 2006). Between 1993 and 2003, the report rate of new smear positive tuberculosis increased from 20 cases per 100000 members of the population to 75 (MAKOMBE, 2006). The prevalence of tuberculosis in many parts of the world has stabilised with the exception of Africa, South-East Asia and the Western Pacific region. There are sub-regional differences in the burden of tuberculosis in Africa with southern and eastern parts having the highest per capita burden (MAKOMBE, 2006). In the Southern African region, seven countries report approximately 400 to 700 cases per 100000 members of the population while North Africa has comparatively the lowest tuberculosis burden of less than 65 cases per 100000 members of the population. In the Central African countries, six out of seven countries reported between 100-200 cases per 100000. Most Eastern African countries report less than 200 cases per 100000 members of the population with the exception of Kenya. More than 60% of Western African countries report fewer than 100 cases per 100000 members of the populations (MAKOMBE, 2006).
In South Africa, tuberculosis is the most commonly notified disease and the country was ranked number four in the list of 22 countries that were affected by tuberculosis (LALL and MEYER, 1999; SEMENYA and MAROYI, 2013). An estimated 285,000 cases of tuberculosis are found in South Africa and this is the seventh highest total number in the world and the second in Africa (GREEN et al., 2010). The incidence rate of tuberculosis was reported to be high in mines exceeding 4000 per 100000 members of the population per annum. This is mainly a result of high prevalence of silica dust exposure (SEMENYA and MAROYI, 2013). In 2009, approximately 407000 tuberculosis cases were notified in South Africa, with KwaZulu-Natal and the Eastern Cape provinces accounting for almost 50% of all cases. In South Africa, the tuberculosis and HIV co-infection rate is high, about 58% of those infected with tuberculosis are co-infected with HIV (GREEN et al., 2010; CDC, 2011). In the North West province of South Africa, tuberculosis was ranked fourth as being responsible for death in all genders, and in Mpumalanga the disease was ranked the sixth cause of death of people (SEMENYA and MAROYI, 2013). In Limpopo province, tuberculosis was ranked as the fifth cause of death of people for all races and genders (SEMENYA and MAROYI, 2013). Of the numerous factors responsible for the tuberculosis epidemic in South Africa, late and inadequate detection of tuberculosis cases; and ineffective diagnostic techniques are of utmost causes (BADASI, 2011). Tuberculosis is still a serious infectious disease in South Africa and effective strategies are needed to control it. Figure 1.1 depicts the incidence of tuberculosis in different districts of each region of the country.
Figure 1.1: Number of tuberculosis cases reported by district in South Africa, 2008. EC, Eastern Cape; FS, Free State; GP, Gauteng province; KZN, KwaZulu-Natal; LP, Limpopo province; MP, Mpumalanga province; NC, Northern Cape; NW, North West, WC, Western Cape (WHO, 2009b).
1.7.5. Modern treatment of tuberculosis

Before the advent of modern tuberculosis chemotherapy, therapeutic strategy was based on bed rest, surgical removal and isolation of the effected part of the lungs. However in 1952, due to research and extensive clinical trials done for many years, the modern treatment for tuberculosis was discovered. The beginning of tuberculosis chemotherapy showed the importance of nature in fighting against the diseases (DE SOUZA, 2009). Tuberculosis treatment was not only effective for curing the disease, but is among the most cost effective ways of prolonging healthy human life (GRANGE and ZUMLA, 2002). After the discovery of streptomycin several synthetic drugs were introduced to the market (DE SOUZA, 2009).

There are two stages of tuberculosis treatment and these are the first line and second line anti-tubercular drugs (WHO, 2006). The drugs commonly used for tuberculosis treatment include rifampin, isoniazid, pyrazinamide, ethambutol, kanamycin and fluoroquinolones which are often used in combination (LAI et al., 2011).

1.7.5.1. First line anti-tuberculosis therapy

The first line anti-tuberculosis drugs include isoniazid, rifampin, pyrazinamide and ethambutol that are used in combination based after considering the needs of an individual patient, sputum culture and sensitivity test results (ARCANGELO and PETERSON, 2006). Using the first line anti-tuberculosis drugs in combination is the single most important factor to be considered when deciding on a tuberculosis treatment, except in the case of latent tuberculosis (SENSI et al., 1959). It is recommended that patients must receive four drugs on a regular basis until culture and sensitivity testing is complete. Isoniazid and rifampin are the pillars of tuberculosis therapy as they have bactericidal effects to both rapidly growing and replicating Mycobacterium tuberculosis organisms. Rifampin (Figure 1.3) has a broad spectrum of activity acting on both the extracellular and intracellular bacilli. The mode of action of rifampin is to inhibit bacterial deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase by blocking the initiation chain formation of RNA (SENSI et al., 1959). Isoniazid (Figure 1.2) is used in both prevention and treatment of tuberculosis. This antibiotic is only bactericidal when there is rapid growth of mycobacteria, but bacteriostatic in the case of slow growing bacteria (AHMAD et al., 2009). Rifampin must be activated by
KatG, a bacterial catalase-peroxidase enzyme to *Mycobacterium tuberculosis*. Isonicotinic acyl and NADH are coupled by KatG to form an isonicotinic acyl-NADH complex that tightly binds to the enoyl-acyl carrier protein reductase, thereby blocking the action of fatty acid synthase and the natural anoyl-AcpM substrate. This process inhibits mycolic acid synthesis in the mycobacterial cell and that leads to death of the bacteria (AHMAD et al., 2009). Pyrazinamid (Figure 1.4) is a bacteriostatic drug, but can be bactericidal on replicating tuberculosis bacteria. It is a prodrug that is activated to pyrazinoic acid by the pyrazinamidase enzyme of *Mycobacterium tuberculosis*. This drug is only active at acidic pH ranging between 5.5 to 5.8, and under normal culture conditions shows little or no activity (LORIAN, 2005). Pyrazinamide is for the initial treatment of active tuberculosis for both adults and children, and should be used in combination with other antibiotics. Ethambutol (Figure 1.5) is a bacteriostatic drug and works in combination with other drugs such as isoniazid, rifampin and pyrazinamide. This drug works by obstructing cell wall formation (ARCANGELO and PETERSON, 2006).
1.7.5.2. Second line anti-tuberculosis therapy

When tuberculosis strains show no sensitivity to the first line drugs, then other antibiotics are recommended. However, before the recommendations to take second line drugs are made, the patient must be monitored by a specialist (Arcangelo and Peterson, 2006). Second line drugs include fluoroquinolones and kanamycin. Fluoroquinolones are important antimycobacterial agents that are used in cases of resistance to first line tuberculosis drugs. It inhibits synthesis of bactericidal DNA through bacterial topoisomerase inhibition ii (DNA gyrase) and topoisomerase iv inhibition, which are responsible for super coiled DNA relaxation and replicated chromosomal DNA separation. Fluoroquinolones eradicate bacteria by interfering with DNA replication, and are ineffective against intracellular pathogens. There are different types of fluoroquinolones, they include ciprofloxin, levofloxacina, gatifloxacina, moxifloxacina, ofloxacina and norfloxacina (Hooper, 2001). Kanamycin (Figure 1.6) is produced during fermentation of Streptomyces kanamyceticus. This drug interferes with protein synthesis in bacterial cells. The mechanism of action is that defective protein synthesis results through its irreversible binding to 30S and 50S ribosomal subunits (Waksmundzka-Hajinos and Sherma, 2011).

![Figure 1.6: Kanamycin](image)

As common with other medications, anti-tuberculosis drugs have side effects. For example pyrazinamide, rifampin and isoniazid can induce hepatotoxicity. Liver injury induced by drugs is a long standing cause of concern in tuberculosis treatment and its rate ranges from 5 to 33%. Rifampin may cause renal failure, hemolysis and thrombocytopenia (due to platelets absorbing anti-rifampin antibodies resulting in damage of the platelet) (Lai et al., 2011).
Furthermore, rifampin and pyrazinamide can cause arthalgias which is evident when the patient experiences pain, and has swelling joints. Ethambutol causes retrobulbar neuritis and the symptoms of this are blurred vision, decreased visual acuity, central scotoma and colour blindness. Other side effects of anti-tuberculosis drugs are hypersensitivity reactions such as acneform skin rash or systemic lupus erythematosus (tissue damage to segments of the vascular tissue and various surfaces) these are caused by isoniazid (LAI et al., 2011).

Tuberculosis treatment consists of a combination of anti-tuberculosis drugs, as a single drug would be less capable of killing a typical tuberculosis bacterium in some patients. Nonetheless, this interaction could have positive and negative effects to the patient’s health. Besides tuberculosis drugs having to interact with each other, they also interact with antiretroviral treatment and this makes tuberculosis very difficult to treat when there is an HIV infection (LAI et al., 2011). For instance, when HIV protease inhibitors (antiretroviral) react with rifampin, its serum concentration is decreased by 35 to 92%. Thus, there is a need to find new anti-tuberculosis drugs without or with less side effects (LAI et al., 2011).

As with other infectious diseases, pathogens have developed resistances to drugs known to treat them, and tuberculosis is no exception. Resistance to the first discovered anti-tuberculosis drug was found in the middle of 1950s (NEGI et al., 2009). Many cases of multi-drug resistant (MDR) tuberculosis are treatable by means of prolonged therapy with more expensive and more toxic drugs given under strict supervision (MANN et al., 2008). This raises the cost of therapy as it takes more time to cure the disease (two years and more) than the usual 6 months, furthermore not all patients are cured (MOLINA-SALINAS et al., 2006). Additionally, the spread of tuberculosis in patients infected with HIV is increasing. The directly observed treatment strategy has not been responding according to expectations as the success rate has been reported to be 67% instead of the expected 85% (GANDHI et al., 2006). This, however increases the need to find new drugs that will not only shorten the duration of treatment but will also be active against latent tuberculosis, MDR and extensive drug-resistant (XDR) tuberculosis and be of use to patients infected with HIV. Since the drugs sometimes cannot inhibit a typical tuberculosis pathogen or drug-resistant strain, new treatments must be found.

The known currently available vaccine for tuberculosis is Bacillus Calmette-Guérin (BCG) which is usually used in children for protecting them against extrapulmonary forms of
tuberculosis such as meningitis (ZELADA et al., 2006; LAI et al., 2011). The effectiveness of the vaccine has been proven in clinical trials and case studies to be 70-80% effective against tuberculosis meningitis in children, the most severe form of the disease. However, the efficacy of the vaccine wanes 10 to 15 years post vaccination, thus adults are not protected against pulmonary forms of tuberculosis and may only be protected against primary forms of infection (ZELADA et al., 2006; DOROKHOV et al., 2007; LAI et al., 2011). Only a few cases have shown BCG vaccine affording protection when given to adults (16 years and older) and virtually no data on persons over 35 years old are available.

1.7.6. Measures taken to control tuberculosis worldwide

In almost all countries, there are tuberculosis control programmes that includes the directly observed treatment short course (DOT) (AL-HAJOJ and VARGHESE, 2013). The DOT programme was recommended by the WHO enabling the patients to be monitored daily by a healthcare worker during treatment (AL-HAJOJ and VARGHESE, 2013). In the United States of America, DOT is a standardised approach that is currently based in communities (FLOYD et al., 1997). Directly observed treatment short course has been reported to be cost effective, it is less expensive than hospital admission, and has achieved a high cure rate (FLOYD et al., 1997). Even in very poor African countries, the cost of hospital care is unlikely to decrease enough to make DOT treatment comparatively inexpensive. However in a study that was done in KwaZulu-Natal, South Africa by FINLAY et al. (2012), half of all patients interviewed reported to be unsupervised when taking their medication. Poor programme implementations were observed more especially where there were high case loads (FINLAY et al., 2012). Previous research noted the delay of tuberculosis treatment by rural patients due to being unable to travel to centres of treatment daily as they could not afford to (MOORE et al., 2009). Some patients often change their living places or experience side effects of drugs or are not knowledgeable about the treatment course. This makes DOT services implementation a challenge and may be an ineffective strategy for tuberculosis control in South Africa. The national coordination of this programme has been improving with greater visibility of political leaders raising support and awareness on tuberculosis (MAKOMBE, 2006). In April 2001, 16 African heads of state and governments showed their commitment in the Abuja declaration and plan of action on HIV/AIDS, tuberculosis and other related infectious diseases to develop strategies that will ensure
adequate prevention and cure of the diseases (MAKOMBE, 2006). However, despite this improvement the rates of case detection and treatment are still low, because DOT services are still not accessible to all segments of the population (MAKOMBE, 2006). Although the DOT remains essential for controlling tuberculosis, according to global statistics the strategy alone will not be enough to achieve the 2015 “stop tuberculosis target” especially on the African continent (MAKOMBE, 2006).

1.7.7. Overview of medicinal plants in treating tuberculosis

Antimycobacterial activity has been tested on a large number of plants worldwide, this is evident from the reports on in vitro inhibition of different species of mycobacteria that have been produced, however, more plants still need to be investigated for their efficacy (LALL and MEYER, 1999; MOLINA-SALINAS et al., 2006; GREEN et al., 2010; LUO et al., 2011). Mycobacteria species were found to be sensitive against extracts of *Maerua edulis* Gilg & Gilg-Ben. DeWolf, *Securidaca longipendunculata* Fresen., *Zanthoxylum capense* (Thunb.) Harv and *Tabernaemontana elegans* Stapf (MOLINA-SALINAS et al., 2006; LUO et al., 2011). In South Africa, research on documenting and evaluating medicinal plants that are used for treating tuberculosis and its related symptoms has been done. Such research includes ethnobotanical studies that were conducted in Limpopo by GREEN et al. (2010), and in rural Maputaland and in KwaZulu-Natal by YORK et al. (2011) to select medicinal plants used in treating tuberculosis. Other studies were conducted by MATIVANDLELA et al. (2008), LALL and MEYER (1999), BUWA and AFOLAYAN (2009) screening randomly selected medicinal plants against strains of *Mycobacterium*. In a study by GREEN et al. (2010), out of a total of 21 medicinal plants screened against *Mycobacterium tuberculosis* H37Ra and a clinical strain resistant to first line drugs, four plants showed noteworthy minimum inhibitory concentration (MIC) values. These were *Berchemia discolor* (Klotzsch) Hemsl., *Bridelia micrantha* (Hochst.) Baill., *Terminalia sericea* Burch. ex DC and *Warburgia salutaris* (G.Bertol.) Chiov with a MIC value of 25 µg/ml. *Artemisia afra* Jacq. ex Wild., *Galenia africana* L. and *Drosera capensis* L. exhibited inhibition of *Mycobacterium segmatis* with MIC values ranging from 0.78 to 6.25 mg/ml, respectively (MATIVANDLELA et al., 2008). Extracts from *Drosera angustifolia* F.Muell. and *Galenia africana* were found to have activity against *Mycobacterium tuberculosis* with MIC values of 5 and 12 mg/ml (MATIVANDLELA et al., 2008). The acetone extracts of *Euclea natalensis*
A.DC. exhibited some activity against *Mycobacterium bovis* with MIC value of 26 µg/ml (McGAW et al., 2008b). The acetone extracts of *Euclea natalensis*, *Helichrysum melanacme* DC. and *Polygala myrtifolia* L. were found to have a MIC value of 0.1 mg/ml (LALL and MEYER, 1999). According to ELDEEN and VAN STADEN (2008) the ethanol (EtOH) extracts of *Acacia seyal* Delile, *Combretum krausii* Hochst., *Kigelia africana* (Lam.) Benth. and *Ziziphus spina-christi* (L.) Desf. showed MIC values between 0.19 and 1.56 mg/ml against *Mycobacterium aurum*. *Coleonema album* (Thunb.) Bartl. & H.L.Wendl. was reported to have activity against *Mycobacterium smegmatis* in the bioautography assay; and the acetone extract from this plant showed antimycobacterial activity against drug sensitive *Mycobacterium tuberculosis* inhibiting more than 99% of the bacterial population at a concentration of 1 mg/ml (McGAW et al., 2008a).

Compounds with antimycobacterial activity have been isolated from plants (FYHRQUIST et al., 2014). Thus, drugs used in tuberculosis therapy might be found from medicinal plants that are used for tuberculosis and other bacterial infection treatments (NEGI et al., 2009). The plant derived compounds with antimycobacterial activity, include aegigerin isolated from *Clavija procera* B.Ståhl which demonstrated good activity against *Mycobacterium tuberculosis* H37Rv and MDR tuberculosis strains (EARL et al., 2010). Diospyrin isolated from *Euclea natalensis* showed activity against a strain of *Mycobacterium tuberculosis* with an MIC value of 100 µg/ml in a study done by LALL and MEYER (2001). Oleanolic acid was isolated from *Buddleja saligna* Willd., and both oleanolic acid and ursolic acids isolated from *Leysera gnaphalodes* (L.) L. showed good antimycobacterial activity when tested against *Mycobacterium avium*, *Mycobacterium scrofulaceum*, *Mycobacterium microti* and *Mycobacterium tuberculosis* H37Rv. The flavone, 5,7,2-trihydroxyflavone isolated from the EtOH extract of *Galenia africana* was found to have activity against *Mycobacterium segmatis* and *Mycobacterium tuberculosis* with MIC values of 0.031 mg/ml and 0.10 mg/ml respectively (MATIVANDLELA et al., 2008). A new compound, termilignan B and arjunic acid isolated from *Terminalia sericea* were reported by ELDEEN et al. (2008) to have displayed insignificant activity against *Mycobacterium aurum* A+. The naphthoquinone 7-methyljuglone and diospyrin isolated from chloroform extract of *Euclea natalensis* (root) showed activity at MIC values ranging from 0.5 to 8.0 µg/ml against drug sensitive and resistant *Mycobacterium tuberculosis* strains (McGAW et al., 2008b). Thus, screening of diverse South African plants for antimycobacterial activity offers much potential in the search
for new active metabolites that may have activity against tuberculosis and other opportunistic infections.

1.8. Medicinal plant trade

Due to the healing role attributed to medicinal plants, many cities have become centers of demand for plants from rural areas and across the borders of South Africa. A major source of income in Africa are medicinal plants. They are not only traded locally but are also widely exported in bulk for the international market. In terms of export figures, the African continent comes second to Asia (ICS-UNIDO, 2004). It was reported by ICS-UNIDO (2004) that more than 10% of the African region’s floristic diversity is likely to be exploited for commercial purposes and half of it is underutilised at present.

There are relatively few plants in South Africa that are utilised economically despite the enormous plant species richness (COETZEE et al., 1999). However, attempts have been made to use plants in boosting the economy of the nation with permission from the legal owners. Most households in South Africa spend between 4-8% of their annual income on TM and services (MANDER, 1998). BOTHA et al. (2004) expressed the opinion that the actual economic value of medicinal plant trade in South Africa may be more than estimated by various researchers, the reason being that in informal trade the volume of sales are not quantified easily due to lack of records and the often illegitimate nature of the trade.

In South Africa, medicinal plant traders conform to two generalised market systems: a primary informal and more formal market (MANDER and MCKENZIE et al., 2005). The marketing is driven primarily by largely informal economics and dominated by simple technologies enabling a wide range of community members to be involved in various trade aspects. The major players involved in harvesting and trading of medicinal plants for the informal sector are rural people, especially women and children (MANDER, 1998). A large informal trade business has been established with medicinal plants as a result of urbanisation. Herb traders trading from fixed licensed premises called herbal muthi shops are representatives of the formal market and this includes phytomedicine, nutraceuticals and cosmeceuticals (COETZEE et al., 1999). Economical exploitation of natural plant resources
is limited, with only the indigenous industry that has managed to successfully establish small and medium scale entrepreneurs.

During the last 10 years, research on southern African medicinal plants has been gaining momentum (VAN WYK, 2008). About 38 indigenous plant species have been commercialised in South Africa and made available in modern packaging and various forms of tea, tinctures, tablets, capsules as well as ointments with several others produced for the multi-million rand informal market (VAN WYK, 2008). In 1998, an estimated 20 000 tonnes of medicinal plants were repeatedly used in TM trade in South Africa. This huge volume has increased pressure on habitats, resulting in many local extinctions (VAN WYK, 2008). Research by in 1997, BOTHA et al. (2004) revealed that approximately 70 different plants representing 40 families were traded in the low-veld of Limpopo province. WILLIAMS et al. (2000) surveyed 50 herbal muthi shops, and reported that 69% of traded medicinal plants were harvested from Gauteng, North West, Limpopo and Mpumalanga. In KwaZulu-Natal, 23 of the 70 traded plants are protected species in the province. In 1998, an estimated 4000 tonnes of plant material was said to have been traded in KwaZulu-Natal. In the same year, Eastern Cape was said to have traded 505 tonnes of plant material (MOENG and POTGIETER, 2011).

1.9. Conservation of medicinal plants

Conservation of medicinal plants involves their preservation and protection, through planned management to prevent neglect, over-exploitation or even destruction (OKIGBO et al., 2008). The income and living standards of rural people that are economically vulnerable in developing countries depends on utilisation and commercialisation of medicinal plants (WILLIAMS et al., 2000). Therefore, wild resource harvesting is an economic activity recognised by developing countries. The demand of medicinal plants whether for scientific evaluation or commercialisation has led to indiscriminate harvesting of the wild flora and has resulted in traditional medicines becoming unavailable to people who have relied on them for years (ROBERSON et al., 2008). As there is continued demand for medicinal plants, there is a need for species conservation through sustainable harvesting and cultivation methods as their overharvesting has placed them at extinction risk (ROBERSON et al., 2008; NJOROGE et al., 2010). According to ROBERSON et al. (2008), about 15000 medicinal
plant species may be threatened with extinction worldwide with experts estimating the loss of at least one potential drug every two years. All these reasons justify the urgent need to study and conserve medicinal plants. Governments and non-profit organisations are expected to act to meet the challenge.

Interest in medicinal plants has been based on the assumption that they will continuously be available for use (ZSCHOCKE et al., 2000). However, slow growing and slow seed reproducing species are more vulnerable to excessive collection as a consequence there are many plants that are threatened to extinction. Medicinal plant management has become an urgent matter because of the rapid increase in informal trade. Destructive harvesting of barks, roots, bulbs and whole plants is of huge concern to resource managers.

Approximately 5400 wild harvested medicinal plant species are utilised in Africa as TM (NAHASHON, 2013). The majority of these medicinal plants are collected from the wild through uncontrolled and unsustainable harvesting. This unsustainable harvesting of medicinal plants has resulted in some species becoming threatened, if not extinct, despite their importance in human healthcare and livelihood. The importance of conserving medicinal plants rose from the cultural, livelihood and economic roles they have in human life (HAMILTON, 2004). However, these resources are disappearing at a disturbing rate, not only because of unsustainable harvesting but also due to natural habitat destruction, thus endangering the lives and livelihoods of the African population as well as the traditional healthcare system (MAUNDU et al., 2006).

The medicinal plants of South Africa are threatened due to uncontrolled harvesting by chopping, ring-barking as well as up-rooting plants, and this has raised a lot of concerns in terms of conservation (MUANALO, 2007). The most commonly utilised medicinal plants in South Africa are trees, from these trees the bark is reported to be used often (ZSCHOCKE et al., 2000). Overharvesting of slow growing bulbous plants result in them being threatened and, they are recognised by the traditional healers as being scarce (MUANALO, 2007). Encouraging traditional healers to collect and use alternative plant parts such as leaves and twigs instead of bark, roots or bulbs could be solutions for these problems.

South Africa does not have devoted laws encouraging connectivity to conservation, however there are several legislations and policies that contain the tools for realising the concept in as
far as biodiversity is concerned (PATERSOON, 2012). These legal tools place restrictions on collection and harvesting of protected resources, such as plants, and prescribe the arrangement of permits for research purposes. According to the EEUSA (2012) 28 legal instruments for conservation of nature exist at provincial level in this region. Many of the laws are out-dated and the nine provinces are at different stages of developing and implementing new ones. These legal instruments generally allow establishment and protection of nature reserves and conservation of threatened species. Examples of some of the laws include the constitution Act 108 of 1996 that recognises the right of South Africans to a healthy, well conserved environment and to benefit from the natural resources economically and socially (EEUSA, 2012). In addition the national environmental Act number 107 of 1998 promotes sustainable use of natural resources and access as well as cooperative governance in environmental management (EEUSA, 2012). The biodiversity Act number 10 of 2004 provides for the management and conservation of South Africa’s biodiversity within the framework of the National Management Act (NEMA) of 1998 that seeks protection of species and ecosystems that warrant national protection (EEUSA, 2012). It also provides for the sustainable use of indigenous biological resources, the fair and equitable sharing of benefits arising from bioprospecting involving indigenous biological resource. In South Africa, there are nature conservation ordinances which place strict limits on the trade of plants (MOENG and POTGIETER, 2011). For example, the Limpopo environmental management Act prohibits any person without a permit to pick, sell, purchase, donate, receive as a gift, be in possession of, import to, export or remove protected plants in the province (MOENG and POTGIETER, 2011). Therefore, through these conservation tools, South Africa is trying to conserve her unique biodiversity and indigenous medicinal plants as they are a valuable resource.

1.10. Aims and objectives

Africa remains the only continent where tuberculosis rates are still increasing (KAMATOU et al., 2007). The problem is worsened by the emergence of drug resistance and coinfection with HIV (WALUSIMBI et al., 2013). Although drugs for treating tuberculosis have been available for many years, due to a number of reasons many patients fail to complete treatment. The reasons that lead to failure to comply with the rules of treatment are that, drugs used have unpleasant side effects, patients are unaware of the consequences of stopping
treatment, standard anti-tuberculosis therapy takes longer (six months), and patients feel better after several weeks of treatment. Patients remain infectious for longer due to poor compliance and are most likely to suffer relapse and die (AL-HAJORI and VARGHESE, 2013). Thus, new treatments from various sources that takes less time and are without side effects are required to alleviate the problems at hand.

The idea that certain plants have healing potential was well accepted a long time ago before the existence of microbes was discovered by mankind (RíOS AND RECIO, 2005). The ethnobotanical studies done in South Africa do not only boost the natural products economic subdivision, but are a means of preserving indigenous knowledge, with medicinal plants playing an important role in both improving health and the livelihood of many indigenous populations. The illnesses that occur as a result of respiratory tract infection such as colds, flu, hay fever, pneumonia, asthma, bronchitis, emphysema, tuberculosis are one of the scope of traditional healing practices in South Africa. Due to the important role that medicinal plants play in the process of drug discovery and development they are recognised as sources of active antitubercular metabolites (McGAW ET AL., 2008A). Thus, the aims of this study were to evaluate the pharmacological properties of plants that are used in South Africa in the traditional treatment of tuberculosis and related symptoms. These tests include antibacterial activity and enzyme inhibition bioassays [cyclooxygenases enzymes (involved in anti-inflammatories)]. Genotoxicity and cytotoxicity evaluation of the plant extracts that showed good antimicrobial activity to test for their safety. This study was also aimed at isolating and identifying compounds from plant extracts that showed good antimicrobial activity. The information obtained from this study could go a long way in validating the use of the plants used in traditional treatment of tuberculosis and related symptoms and offer scientific credence to the ideas of integrating such plants in healthcare systems which in turn will be helpful to the population.

1.11. Plant selection

Ten plants were selected based on available literature of medicinal plants used by various South African tribes in the treatment of tuberculosis and related symptoms (MABOGO, 1990; HUTCHING ET AL., 1996; VAN WYK ET AL., 1997; McGAW ET AL., 2008A; VAN WYK ET AL., 2009). The plant materials were collected from the University of KwaZulu-
Natal (UKZN) Botanical garden and Ukulinga Research Farm. Voucher specimens were deposited in the UKZN (Pietermaritzburg) Herbarium (NU) for botanical authentication if required in future.

1.12. Botanical description and distribution of the plants selected


This plant is known by a variety of names in South Africa. The common names of *Abrus precatorius* subsp. *africanus* in English are coral-bead plant, crab's eye, jecquirty bean, love bean, lucky bean creeper, minnie-minnies, prayer bean, rosary pea, paternostertjie in Afrikaans, umkhokha in Zulu or umuthi wenhlanhla, umuthi wentlahla in Xhosa, nsisani in Tswana and amabope in Ndebele (BARCELOUX, 2008).

![Figure 1.7: Abrus precatorius subsp. africanus](image)

This plant is a perennial climber that twines around trees, shrubs, and hedges without any special organs of attachment (BHUTIA and MAITI, 2011). The plant has slender branches, a cylindrical wrinkled stem with a smooth-textured brown bark and glabrous alternate compound paripinnate leaves with stipules. It bears oblong and obtuse leaflets, and each is
about 12-18 mm long. Both leaf ends are blunt, glabrous on top and slightly hairy below. It has small and pale violet flowers with a short stalk that are arranged in clusters. The fruit (a pod) is silky in texture, flat, oblong and truncate shaped with a sharp, deflexed beak. The pod contains from three to five oval shaped seeds that are about 6 mm long, bright red with smooth, glossy texture and a black patch on top (IWU, 1993). In South African, the plant is found in KwaZulu-Natal, Gauteng, and some parts of the Limpopo province (FODEN and POTTER, 2005a).


The common names of *Asparagus africanus* are wild asparagus in English, Haakdoring in Afrikaans, isigoba in Zulu, ubulawu obumhlope or umthunzi in Xhosa, and Lelala-tau-le-leholo in Sotho.

![Figure 1.8: Asparagus africanus (DEEDI, 2011)](image)

*Asparagus africanus* is a fast-growing plant that can climb up trees and can grow as a scrambling low shrub without a host, with stems that can be up to 12 m in height. It has thorny leaves and stems (UMBERTO QUATTROCCI, 2012). The roots of this plant do not produce tubers. It has small fragrant flowers that are about 5 to 7 mm wide, green-white
and bell shaped (IWU, 1993). Asparagus africanus produces berries that contain one seed. The colour of Asparagus africanus berries is green, but when ripening it turns orange. This plant species is native to southern Africa, and prefers bushland and rainforests (UMBERTO-QUATTROCCHI, 2012). In South African the plant species is found along the Eastern Cape coast to KwaZulu-Natal, Limpopo, Mpumalanga, and Western Cape provinces (VON STADEN, 2012).

1.12.3. Asparagus falcatus (L.) Oberm.

The common names for this plant are thorny creeper in English, doringtou in Afrikaans, imbelekazana in Zulu, and isigoba in Xhosa.

![Figure 1.9: Asparagus falcatus](image)

Asparagus falcatus is an evergreen climbing shrub that grows up to 7 m in height. The older stems are light grey and have sharp, hard thorns that are curved backwards for protection against predators and to grip onto the host plant to reach sunlight. The leaves are sickle shaped, shiny dark green, feature a prominent vein and are up to 8 cm long. It has roots that are swollen tubers resembling sweet potatoes. The flowers are attractive, small, white and
fragrant. The fruit is a red berry and the seeds are round, shiny and black. The plant occurs in the Eastern Cape, through KwaZulu-Natal into the low-veld of South Africa in forest and forest edges of these locations (FODEN and POTTER, 2005b).


*Brunsvigia grandiflora* is commonly known in South Africa as the candelabra flower in English, kandelaarblom in Afrikaans, umqhele-wenkunzi in Zulu, and isichwe in Xhosa.

![Image of Brunsvigia grandiflora](image)

**Figure 1.10: Brunsvigia grandiflora** (McMASTER et al., 2010)

*Brunsvigia grandiflora* is a large perennial bulb that can reach up to 20 cm in diameter (MANNING et al., 2002). The bulb is covered usually with tan coloured and brittle tunics (MANNING et al., 2002). The flowers of this plant are star shaped, 30 mm in diameter, and deep pink in colour. The flower heads are large and can grow up to 40 cm in diameter with 30 to 60 individual flowers on stalks joined together on a thick stem. The leaves are decorative, grey-green, and upright, often spiraled with waxy margins and dry off during flowering (MANNING et al., 2002). When the seeds are dry, the main stem dries and the head falls off, the seeds are blown away by the wind, and scattered across the veld. In South Africa, the
plant is distributed in grasslands of the Eastern Cape, Free State, KwaZulu-Natal and Mpumalanga provinces (SNIJMAN and VICTOR, 2004).

1.12.5. *Ficus sur* Forssk.

The common names for *Ficus sur* are broom cluster fig, bush fig, cape fig, wild fig, fire sticks, kooman fig, malabar tree in English; besembosvy, besemtrosvy, bosvyeboom, grootvy, koeman, suurvy, wildevyeboom in Afrikaans; amakhiwane, ingobozweni, intombi kayibhinci, umkhiwane in Zulu; ikhiwane, umkhiwane, umkwane in Xhosa; mogo, mogotshelo, mphai, mphayi in Sotho; nkuwa xinkuwana in Tswana; muhuyu, muhuyu-lukuse, muhuyu-ngala in Venda; ikuwu, likwani, mouwane, umkhiwa, umkiwa in Ndebele; and umkiwane in Swati.

![Figure 1.11: Ficus sur. Inset shows the fruit of Ficus sur.](image)

*Ficus sur* is a large, semi-deciduous, fast-growing tree with a short trunk. The tree grows approximately 35 m high (VAN WYK et al., 2008). The leaves are alternate, large, oval, green, hairless, tip usually tapering, and borne on a massive, spreading crown. Figs are 20-40 mm in diameter borne in large leafless clusters mostly low down on the trunk and can even appear at ground level arising from the roots. The figs are smooth or slightly hairy orange-red
or red when ripe. This species is widely distributed in the Western Cape in South Africa usually on riverbanks or in riverine forest but also in drier woodlands (BURROWS and VICTOR, 2005).

**1.12.6. Indigofera arrecta** Benth. ex Harv. & Sond.

The common names for this plant are Natal indigo in English, verfbossie in Afrikaans and umphhekambedu in Zulu.

![Indigofera arrecta](image)

**Figure 1.12: Indigofera arrecta** (HYDE et al., 2014)

*Indigofera arrecta* is an erect, woody, large shrub up to about 3 m in height. The leaves are spirally arranged, 2 to 9 mm long, and have about seven to 21 leaflets usually smooth above and hairy beneath (ORWA et al., 2009). Inflorescence is up to 5 cm long but usually much shorter. Flowers are about 5 mm long, pinkish or reddish, brown strigulose outside. The fruit of *Indigofera arrecta* is a linear pod about 12 to 25 mm long and 2 mm wide, brown when ripe and has four to eight seeds. In South African the plant is found in KwaZulu-Natal, Mpumalanga, Gauteng and Limpopo provinces (FODEN and POTTER, 2005c).

The common name of *Leonotis intermedia* in English is the wild dagga plant. *Leonotis intermedia* is a woody shrub.

![Figure 1.13: Leonotis intermedia](image)

This shrub is multi-stemmed, with stems rising from a woody base, unbranched, pale brown in colour and square in section (*SCHMIDT et al., 2002*). The leaves are dark green above and paler below, arranged in opposites, broadly ovate, densely hairy with coarsely toothed margins. It has orange to reddish flowers. This species is distributed in South Africa, and found in the Eastern Cape, KwaZulu-Natal, and Northern provinces (*MANYAMA and KAMUNDI, 2006*).

1.12.8. *Pentanisia prunelloides* Schinz

The common names for this plant are wild verbena and broad-leaved pentanisia in English, sooi-brandbos in Afrikaans and icimamliolo in Xhosa.
*Pentanisia prunelloides* is an erect perennial herb that grows up to about 30 cm in height and 60 cm wide with stout hairy stems sprouting from a tuberous root. It has oblong leaves, that are usually hairy and in pairs (VAN WYK et al., 2009). The flowers of this plant species are small, pale purple occurring in dense groups on the branch ends. This plant is found in South Africa, provinces such as Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga (FODEN and POTTER, 2005d). In Eastern Cape, *Pentanisia prunelloides* is an important part of the grassland (VAN WYK et al., 2009).

**1.12.9. Polygala fruticosa P. J. Bergius**

The common names for *Polygala fruticosa* are featherdusters, heart-leaved polygala, and shrubby polygala in English, or *ithethe* in Zulu (FODEN and POTTER, 2005e).
*Polygala fruticosa* is a shrublet that can grow up to 2 m in height and has numerous erect branches (*VAN WYK et al., 2009*). The leaves are arranged opposite to each other on the stems, lance or heart-shaped and greyish-green in colour. It has purple flowers that are similar to those of the legume family but distinguished by a tufted outgrowth near the tip of the bottom petal. This plant is distributed in South Africa along the coastal parts of Western Cape, Eastern Cape and KwaZulu-Natal provinces (*VAN WYK et al., 2009*).

**1.12.10. Terminalia phanerophlebia Engl. &Diels**

Bastergeelhout in Afrikaans, amangwamhlophe or amangwansundu in Zulu are the common names of *Terminalia phanerophlebia*. 
Terminalia phanerophlebia is a shrub or small deciduous tree that is approximately 3-7 m high with a rounded crown (SCHMIDT et al., 2004). The stem is about 300 mm in diameter with a dark grey-brown to blackish bark. Leaves are fresh green to dull grey green or dark olive green above and paler below, closely placed along stems and are hairy beneath (SCHMIDT et al., 2004). The flowers are small about 4 mm wide, and are white to creamy white in colour. Fruits are held in clusters with a central nut that is surrounded by a hard ribbed wing, with a pale green colour when young and brown or pinkish brown when ripe (SCHMIDT et al., 2004). The distribution of Terminalia phanerophlebia in South African is in northern KwaZulu-Natal and Mpumalanga provinces (FODEN, POTTER, 2005f).
Chapter 2: *In vitro* antimicrobial activity of extracts from plants used traditionally in South Africa to treat tuberculosis and related symptoms

2.1. Infectious diseases

When pathogens such as bacteria, viruses, or parasites invade and multiply in the cells and tissues of a body, an infectious disease may occur. Infectious diseases have been present since antiquity, thus critically shaping the history of mankind (BELTZ, 2011). Evidence of the presence of an infectious disease such as tuberculosis was found on human bones from about 5000 BC with Hippocrates describing a disease characterised by lung nodules matching the tubercles found in tuberculosis in about 1000 BC (BELTZ, 2011). A few decades ago, there was enormous optimism about the decline of life threatening infectious diseases due to advances in technology and science (COHEN, 1998). However, due to a rise in the transitional nature of infectious diseases, such optimism has not yet been met. Worldwide, infectious diseases are still the leading causes of death, especially in developing countries, claiming millions of lives yearly despite the enormous improvements made in human healthcare (YORK et al., 2011). On a global scale, infectious diseases are the second leading cause of death, disabling and disfiguring many lives, thus having a huge negative effect on the human population (HAMBURG, 2008; YORK et al., 2011). This rise in infectious disease mortality is credited to the increase in diarrhoea, respiratory tract infections and HIV/AIDS which account for about half of all deaths (PINNER et al., 1996). The rise in the transitional nature of infectious diseases is the greatest challenge of the present era (HAMBURG, 2008).

Death due to infectious diseases on both children and adults under the age of 50, places a severe burden on developing parts of the world (HAMBURG, 2008). There has been an emergence of 30 life threatening infectious diseases that have been discovered within the last few decades with the majority being zoonoses or human diseases (HAMBURG, 2008). A few examples of such infectious diseases are HIV, acute respiratory syndrome, H5N1 avian influenza, Ebola and the Nipah virus. Re-emergence of old diseases like WestNile disease, monkeypox, dengue fever, malaria and tuberculosis more especially in new geographic regions have been witnessed (HAMBURG, 2008). Almost all nations have been affected by
the emergence or reemergence of infectious diseases such as malaria and tuberculosis that have occurred in new and more dangerous forms that are resistant to drugs. Presently this is a serious world health concern (HAMBURG, 2008).

Several factors have contributed to the explosion in emerging as well as reemerging infectious diseases worldwide, and these include increasing numbers of immunocompromised individuals and microbial evolution. Many of the emerging diseases appear new to humankind, whereas reemerging ones represent an increasing threat (BELTZ, 2011). Tools such as the discovery of antimicrobials and a better understanding of infectious diseases have been developed to prevent and cure illnesses. This had decreased the incidence and severity in developed countries. However, this is again increasing worldwide (BELTZ, 2011). Overcrowded conditions such as slums of urban areas, homeless shelters, prisons and nursing homes, contribute to the rapid spread of infectious diseases such as tuberculosis. Addressing the problem of infectious diseases is now an important and urgent requirement.

2.1.1. Bacterial infections

Bacteria are microscopic unicellular organisms that are able to adapt and live in different ranges of environments such as soil, water, air and even in bodies of other organisms (SLEIGH and TIMBURY, 1998). They are one of the most abundant group of organisms on earth and can either be beneficial or harmful. These micro-organisms are prokaryotes that reproduce by fission or forming spores. Major forms of bacteria can be recognised as spheres, rods (bent or curved rods) as well as spirals and they are nearly colourless and transparent with a refractive index that is close to that of the surrounding liquid (RYAN and RAY, 2004). Based on whether the organisms do or do not stain with Gram's stain, bacteria can be classified generally as either Gram-positive or Gram-negative (LEE and SCHNEENWIND, 2001). When bacterial cells are stained with crystal violet, Gram-positive’s are those that retain the crystal violet after being decoloured with alcohol, treated with safranin and washed in water. Those that do not retain the dye are Gram-negative. Gram-positive and Gram-negative bacteria differ from each other in many ways and they can be broadly differentiated based on the structure of their cell walls. The function of the cell wall is to provide structural support, protection against osmotic damage and act as a filtering mechanism determining what enters and leaves the cells.
Gram-positive bacteria have a relatively simple cell wall structure that is composed of peptidoglycan polymers cross-linked by polypeptides to form a rigid protective coat (SLEIGH and TIMBURY, 1998). They also have a single plasma membrane followed by a thick cell wall layer that functions as a surface organelle for carbohydrate and protein display (O’LEARY and CAPOTE, 2008). Gram-negative bacteria have a more complex cell wall structure with an inner wall that is interposed between the outer and inner membrane (RANG and DALE, 1987). Both the inner and outer membranes have a characteristic lipid bilayer, but the outer membrane has a lipopolysaccharide component that permits or excludes certain macromolecules.

Human beings are infected with, and are constantly exposed to bacteria. Most bacterial strains that humans are exposed to on a daily basis are not harmful, constitute the commensal flora, and are beneficial (HERITAGE et al., 1999). Only a few bacterial strains interact with humans to cause disease, and they can cause a wide range of different infections ranging from inapparent to fulminating, causing infections to be widespread (AVILA et al., 2008). In immunocompromised patients, normal bacterial strains that are known to be harmless often cause opportunistic infectious diseases (MACKOWIAK, 1982). Examples of common bacteria found in the upper respiratory tract that are part of the normal flora include Staphylococcus aureus, Staphylococcus epidermis, Haemophilus species, Neisseria species, Corynebacterium species, Propiorubacterium acnes, and many anaerobes such as Bacteriodes as well as members of the Enterobacteriaceae (HERITAGE et al., 1999). Although, bacteria from the upper respiratory tract are washed downwards towards the lower respiratory tract, the action of the ciliated epithelium and sticky mucus that covers the lining of the bronchial tubes keeps the lower respiratory tract free of these microorganisms (MACKOWIAK, 1982). The relative pathogenicity of a bacterium to cause disease depends on its capacity. Pathogens can be opportunistic, for example the ones isolated from immunocompromised individuals, or they may be agents of disease just like in the case of Escherichia coli (PETERSON, 1996). Worldwide, approximately 17 million deaths per annum are reported to be caused by bacterial infections especially in children (DEMAIN and SPIZEK, 2012). Bacterial infections of the lower respiratory tract include Streptococcus pneumoniae, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas species, Legionella pneumophila, Mycoplasma pneumoniae, as well as Chlamydia pneumoniae. These are known to infect HIV/AIDS patients. However, in sub-Saharan Africa, the most common cause of respiratory tract bacterial infection in HIV-infected patients is tuberculosis, although
other infections also occur, this is the main source of concern (BATUNGWANAYO et al., 1994).

2.2. Drug-resistance

Infectious disease treatment is currently facing a major challenge, with many microbes developing resistance to widely used antibiotics. Both Gram-negative and Gram-positive bacteria have acquired resistance to antimicrobial drugs used worldwide (WILSON et al., 2002). This in turn has led to a rise in morbidity, mortality and cost of healthcare (DE SOUZA, 2009). Drug-resistance is defined as the ability of an organism to withstand antibiotic treatment by either making it inactive or less effective.

Antimicrobial resistance mechanisms in bacteria can either be natural or acquired (WILSON et al., 2002). Natural resistance entails that the bacteria have ‘intrinsic’ resistance; for instance, some bacterial strains have genes that cause resistance to an antibiotic known to kill them, while others lack transport systems or targets for antibiotics (GEORGIEV and FAUCI, 2009). Acquired resistance refers to bacteria that usually show sensitivity to antibiotics, but are likely to develop resistance, and this occurs through spontaneous gene mutation, or mobile genetic element acquisition, which carry the antibiotic resistance genes (WILSON et al., 2002). A mutation altering the binding site of a drug decreases antibiotic sensitivity, thus increasing drug-resistance. Resistance of Mycobacterium tuberculosis to streptomycin is an example of such a kind of mechanism (WILSON et al., 2002). There are a lot of bacterial strains that have acquired antimicrobial resistance, and they include respiratory pathogens such as Klebsiella pneumoniae, Staphylococcus aureus and Mycobacterium tuberculosis (WILSON et al., 2002). Resistance of bacteria to drugs is linked to usage of antimicrobial agents in clinical practice (GIEDRAITIENĖ et al., 2011). The use of therapeutic antibiotics for a long time can result in microorganisms that showed drug sensitivity prior to the development of resistance through adaptation. Resistance of Staphylococci species to penicillin marked the beginning of antimicrobial resistance.

The past two decades have seen the emergence of pathogenic infectious diseases, such as AIDS and drug-resistant tuberculosis which represent a substantial global threat to human health. Tuberculosis is presently one of the most serious bacterial infectious diseases that
causes a threat to healthcare of humans globally (CHEN et al., 2011). There has been an emergence of resistant tuberculosis strains to first line antibiotics known as single drug-resistant, MDR tuberculosis and XDR tuberculosis (WHO, 2009a). It is estimated by the WHO that 50 million people may be infected with drug-resistant tuberculosis worldwide (WHO, 2009a). Additionally, in the next few years emerging diseases will probably increase due to travel, urbanisation, overcrowding, inadequate healthcare, continuing globalisation of trade leading to new interactions between human beings and animals as well as other disease vectors (HAMBURG, 2008; ALIROL et al., 2011). Thus, addressing the problems of infectious diseases now is an important and urgent requirement (HAMBURG, 2008).

2.2.1. Multi drug-resistant tuberculosis

Multi drug-resistant tuberculosis is highly contagious and it is due to resistance to rifampin and isoniazid (SINGH et al., 2011). Multi drug-resistant tuberculosis was recognised by the WHO-International Union against tuberculosis and lung disease in nearly every country surveyed from 1994 to 2000 as part of the global drug-resistance surveillance project (SINGH et al., 2011). According to surveys from 184 countries, 458000 new MDR tuberculosis cases occurred worldwide in 2003 and out of these cases, 60% were reported from China and India constituting 3.2% of all new tuberculosis cases (SINGH et al., 2011). In 2012, a report was issued by the WHO which stated that China, India, Russian and South Africa represents 60% of the total cases of MDR tuberculosis, thus referred to as the burden countries out of 27 nations (SOTGIU et al., 2013). Multi drug-resistant tuberculosis is widespread and is recognised as a threat to tuberculosis control as it may result in death unless treated quickly and effectively (ALEXANDER and DE, 2007). It is estimated that at least 4% of tuberculosis patients have MDR tuberculosis, with 40% of them having been previously treated for tuberculosis (ALEXANDER and DE, 2007). For a person with MDR tuberculosis short term chemotherapy is ineffective and its treatment requires multidisciplinary care for more than a year (ALEXANDER and DE, 2007). The situation of increasing incidence of MDR tuberculosis worldwide, highlights the urgent need to search for newer anti-tuberculosis agents or drugs. Figure 2.1 shows a report by the WHO (2013) on new global MDR tuberculosis cases in 2012.
2.2.2. Extensive drug-resistant tuberculosis

Due to improper treatment and management, XDR tuberculosis emerged, which is defined as resistance of pathogens to first line antibiotics as well as fluoroquinolones and at least 1 of 3 injectable second-line drugs such as capreomycin, kanamycin or amikacin (DE SOUZA, 2009). The outbreak of XDR tuberculosis was observed for the first time in September, 2006; in a rural hospital in Tugela Ferry, South Africa where 53 cases were diagnosed, out of which 52 patients died (SINGH et al., 2011). Among four countries estimated to have the leading number of XDR tuberculosis, South Africa is included (FYHRQUIST et al., 2014). There is growing proof that drug-resistant tuberculosis is transferred from one person to the next, thus supporting the conclusion that the estimated number of cases here in South Africa are only a portion of a larger phenomenon. Extensive drug-resistant tuberculosis is threatening to make the disease untreatable, more especially in the case of co-infection with HIV. All South African provinces have reported cases of XDR tuberculosis, since the Tugela Ferry outbreak was discovered, and Western Cape in particular has a very high rate of tuberculosis bacterial strains resistance to drugs (OLSON et al., 2011). It has been reported that throughout KwaZulu-Natal, there are MDR and XDR tuberculosis cases, thus causing the province to
have an enormous tuberculosis burden (Olson et al., 2011). The latest estimations have reported that more than 60 facilities in KwaZulu-Natal have at least one case of XDR tuberculosis (Olson et al., 2011).

The disturbing epidemiology of MDR and XDR tuberculosis strains and difficulty in treating the disease that is spreading throughout South Africa creates an urgent reason for solving the problem. According to the WHO, this is a new serious global public health problem (De Souza, 2009). In fact, reports from all over the world have shown that XDR tuberculosis treatment outcomes are poor. It is likely that more resistant strains of Mycobacterium that will exhaust the current chemical defence that are available at present will emerge in the future. Therefore, there is need for new and alternative ways of counteracting this problem.

2.3. Ethnopharmacological approach to drug resistance problem

Ethnopharmacology is a specially designated field of science defined as the evaluation of natural products from medicinal plants, to expand their knowledge base in order to further develop the use of this information (Heinrich and Gibbons, 2001). The evaluation of medicinal plants is done by observing, identifying, describing and further conducting experimental investigations of the ingredients and their effects. Thus, making ethnopharmacology a truly interdisciplinary field of research which is very important in the study of TM (Leonti, 2011). Ethnopharmacology in a modern sense, only became possible with the scientific ability to study the effects of substances and extracts on model systems (Heinrich and Gibbons, 2001). Ethnopharmacological approaches have generally followed a practical approach and are aimed at the experimental investigation and biological validation of medicinal plants from traditional ethnic groups (Leonti, 2011; Novaes and Leite, 2011). Thus, the broad perception of ethnopharmacology contextualises ecology and addresses the perception of plants, plant use, pharmacology and physiology in human communities.

Which method to use when selecting plants for biological evaluation is one of the main challenges at present, because this process is the first research step. Various methods are used in selecting plants for pharmacological evaluation. These methods include: random selection of the species without consideration of taxonomic and ethnobotanical information (Novaes
and LEITE, 2011); ethnodirected selection, which encompasses ethnobotanical and ethnopharmacological approaches and applies information about the traditional use of plants to treat specific diseases; chemotaxonomic approaches that are based on studying plants from the same family or genus of a species from which bioactive secondary metabolite have been isolated already (SILVER et al., 2013). However, an ethnopharmacological approach in selecting plants has previously shown to be advantageous towards discovering plants with compounds that have pharmacological properties (NOVAES and LEITE, 2011). In bioprospecting, medicinal plants are selected based on the idea that they are used for treating specific illnesses and may have an associated biological activity (AHMAD et al., 2006).

2.4. Bacterial strains related to respiratory ailments that were selected in this study

Conducting research on anti-tuberculosis plants against MDR and XDR tuberculosis strains is rather a challenge, due to the fact that growth of *Mycobacterium tuberculosis* is slow and that the bacteria is highly dangerous to work with and requires strict biosafety conditions. All these problems pose considerable obstacles, but in alleviating the critical issues at hand, non-pathogenic fast growing mycobacteria, like *Mycobacterium aurum* have been introduced as test organisms in the drug screening process. Additionally, among non-pathogen strains of *Mycobacterium*, *Mycobacterium aurum* is the closest to *Mycobacterium tuberculosis* in terms of mycolate components (GUPTA et al., 2009). The structural similarities in mycolate components are responsible for cell wall envelope permeability to antimicrobials, thus the use of *Mycobacterium aurum* in anti-tuberculosis research is recommended. The ability of this strain to survive in an intracellular environment is another advantage for the use of this bacterial strain for the second step of drug screening.

2.4.1. *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is a Gram-negative, aerobic, non-motile, rod shaped, lactose fermenting bacteria. This bacterium is the most prevalent and clinically important bacterial strain found in normal flora of the mouth, skin and intestines of humans. It has cells that are surrounded by a gelatinous capsule inhibiting phagocytosis and produces large, moist and often very mucoid colonies (DAVIS et al., 1990). Infection due to *Klebsiella pneumoniae* if
not treated can cause destructive changes to human lungs and often becomes fatal (PRICE and FREY, 2003). *Klebsiella* infections are seen mostly in people with a weakened immune system, most often illness affects middle-aged and older men with debilitating diseases. *Klebsiella pneumoniae* tends to affect people with diabetes or chronic pulmonary disease. In the USA, approximately 3% of all bacterial pneumonia whether nosocomial or community acquired are caused by *Klebsiella pneumoniae* (PRICE and FREY, 2003). Infection with *Klebsiella pneumoniae* can also cause mild respiratory tract infections such as bronchitis and sometimes lobar pneumonia, and meningitis. *Klebsiella pneumoniae* is thus, a secondary invader in the lungs of humans suffering from chronic pulmonary disease (PRICE and FREY, 2003). The bacterium is highly resistant to antibiotics attributable to acquiring plasmids and changes to its chromosomal gene structure (PRICE and FREY, 2003).

### 2.4.2. *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive, facultative anaerobe that grows rapidly and abundantly under aerobic conditions as grape like-structures. Species of the genus *Staphylococcus* are prominent inhabitants of the human respiratory tract and skin, and may be highly pathogenic when they invade the underlying tissues (FUERST, 1978). *Staphylococcus aureus* is a dangerous coagulase producing bacteria that typically produces boils and carbuncles. This bacterial strain can invade the human system, causing fatal septicemia, meningitis, endocarditis, puerperal sepsis, pneumonia, multiple abscesses and osteomyelitis. The strain is of special importance in hospitals, causing epidemics of antibiotic resistance, and can therefore remain alive for several months under favorable conditions.

### 2.4.3. *Mycobacterium aurum*

*Mycobacterium aurum* is a fast growing, non-infectious, Gram-positive bacterium. This bacterium belongs to a different category of mycobacteria that do not cause tuberculosis (HONARVAR et al., 2012). This bacterial species also called non-tuberculous mycobacteria (NTM), is also known as an environmental mycobacteria since it is particularly found in the environment, mostly in wet soils, rivers, lakes, and swamps. In contrast to typical tuberculosis infections, this mycobacterial infection is non-communicable. The high level of resemblance in the susceptibility profile of cell wall inhibitors in *Mycobacterium aurum* and
Mycobacterium tuberculosis, makes it a recommended organism for use in the search for new inhibitors. The ability to survive in an intracellular environment is another advantage for using Mycobacterium aurum for drug screening (GUPTA et al., 2009).

2.4.4. Mycobacterium tuberculosis H37Ra

Mycobacterium tuberculosis H37Ra is a non-motile, rod-shaped bacterium and one of the causative agents of tuberculosis. This bacterium is the avirulent counterpart of the virulent strain H37Rv derived from H37 which is a virulent parent strain, and was originally isolated by Edward R. Baldwin in 1905 from a 19 year-old male patient with chronic pulmonary tuberculosis (ZHENG et al., 2008). H37Ra (“a” for avirulent) has been widely used as a reference strain for studying virulence and pathogenesis of Mycobacterium tuberculosis worldwide since 1940s and is also used as an adjuvant to boost immunogenicity during immunisation (ZHENG et al., 2008). A large part of the success of this pathogen is due to its ability to persist in a dormant or latent form for years without showing clinical symptoms. Despite being considered as Gram-positive, mycobacteria have an unusual outer membrane approximately 8 nm thick. The outer membrane and the mycolic acid-arabinogalactan-peptidoglycan polymer form the cell wall and constitutes an efficient permeability barrier in conjunction with the cell inner membrane.

The traditional use of medicinal plants has been documented extensively in many regions of South Africa by various researchers. This is evident from the books written by WATT and BREYER-BRANDWIJK (1962); HUTCHINGS et al. (1996) and VAN WYK et al. (1997 and 2009). Though, the effectiveness of the plant preparations may be reported by traditional practitioners, their scientific validation is required for confirmation. Most studies done in South Africa on plants used traditionally to treat respiratory ailments have focused only on evaluating their antimycobacterial activity against tuberculosis-causing bacterial strains (LALL and MEYER, 1999; GREEN et al., 2010; MATIVANDLELA et al., 2008) but with little emphasis on the related symptoms (BUWA and AFOLAYAN, 2009; YORK et al., 2012). Therefore, this current study was aimed at evaluating the antimicrobial activity of selected plants against Mycobacterium species and other bacterial strains related to respiratory infection.
2.5. Materials and methods

2.5.1. Preparation of plant extracts

Immediately after collection, plant materials were oven dried at 50 ºC. The dried plant materials were ground into fine powders using an ultra centrifugal mill (ZM 200, Retsch® Germany) and stored in airtight containers. Dried plant material (10 g) was extracted sequentially with 200 ml of each of four solvents; in the following order petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water (from non-polar to polar) by sonication for 1 h in an ice water bath. Organic extract samples were then filtered through Whatman No. 1 filter paper and concentrated to dryness under reduced pressure using a rotary evaporator to obtain crude extracts. The aqueous extracts were freeze-dried under reduced pressure. Crude extracts were then stored in the dark at 10 ºC until use. The percentage yields for each extract were determined.

2.5.2. Bacterial strains and culture conditions

Four bacterial strains were used in this study to evaluate the plant extracts. *Klebsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus* (ATCC 12600), *Mycobacterium tuberculosis* H37Ra (ATCC 25177) obtained from the American Type Culture Collection (ATCC), and *Mycobacterium aurum* A+ from the Microbiology Laboratory, Division of Pharmacology, University of Cape Town were used for the assay. The test organisms were selected because of their respiratory pathogenesis (ELDEEN and VAN STADEN, 2007; BUWA and AFOLAYAN, 2009; GREEN et al., 2010; YORK et al., 2012). The strains were maintained on Mueller-Hinton (MH) (Merck, South Africa) agar except for *Mycobacterium aurum* A+ and *Mycobacterium tuberculosis* H37Ra that were maintained on Middlebrook 7H10 agar (Sigma-Aldrich, Steinheim) supplemented with either 0.5% or 0.2% glycerol and 10% OADC (oleic acid, albumin, dextrose, and catalase; Sigma-Aldrich, Steinheim).

2.5.3. Procedure for microorganism long term storage

Prior to storage, MH agar (Merck, South Africa) and Middlebrook 7H10 agar supplemented with 0.5% or 0.2% glycerol and 10% OADC were prepared, sterilised and poured (25 ml)
into sterile plastic Petri dishes and allowed to gel. After gelling of the agar, the Petri dishes were kept at 4 °C.

The Kwik-stik™ unit was removed from 2-8 °C storage and allowed to equilibrate to room temperature. The middle of the ampoule cap of the Kwik-stik™ unit was pinched to break the ampoule and that was followed by release of the hydrating fluid which was then allowed to flow through the swab shaft and into the bottom portion of the unit containing the gelatine pellet. The bottom portion of the pellet was crushed by pinching it repeatedly to form a homogeneous suspension. The fluid-hydrated swab was saturated and transferred to Petri dishes with MH agar (Klebsiella pneumoniae and Staphylococcus aureus) or Middlebrook 7H10 agar supplemented with 0.5% or 0.2% glycerol and 10% OADC (Mycobacterium aurum A+ and Mycobacterium tuberculosis H37Ra). For each bacterial strain, the same swab was used repeatedly to streak the inoculated area. The inoculated agar plates were then incubated for 24 h (Klebsiella pneumoniae, Staphylococcus aureus), or 72 h (Mycobacterium aurum A+) at 37 °C to allow bacterial colonies to grow. For Mycobacterium tuberculosis H37Ra, the agar plates were incubated in a 5% carbon dioxide incubator at 37 °C for four weeks.

After incubation, the bacteria were kept as stocks in sterile cryovials (Greiner, Germany) containing glycerol solution for Klebsiella pneumoniae and Staphylococcus aureus. Whereas Mycobacterium aurum and Mycobacterium tuberculosis H37Ra stocks were kept in sterile Middlebrook 7H9 broth supplemented with a 0.5% glycerol and 10% OADC supplement. The cryovials with stock cultures were then stored in an ultra-freezer at -70 °C until required for antibacterial assay.

2.5.4. Subculturing for bioassays

Bacterial stock strains were streaked and sub-cultured in sterile MH agar (for Klebsiella pneumoniae and Staphylococcus aureus) or Middlebrook 7H10 agar supplemented with 0.5% glycerol and 10% OADC supplement (for Mycobacterium aurum and Mycobacterium tuberculosis). The plates were incubated for 24 h for Klebsiella pneumoniae and Staphylococcus aureus, 72 h for Mycobacterium aurum and 3 to 4 weeks for Mycobacterium tuberculosis H37Ra to allow bacterial colonies to develop. When the bacterial colonies had developed, the plates were then stored at 4 °C to prevent further bacterial growth. The same
procedure was followed to sub-culture stocks on a monthly basis in order to maintain viability.

2.5.5. Antibacterial activity using microdilution

The minimum inhibitory concentrations (MIC) of plant extracts were determined using the microdilution method in 96 well microtitre plates against *Staphylococcus aureus* and *Klebsiella pneumoniae* as described by ELOFF (1998a). The extracts were dissolved in 10% dimethylsulfoxide (DMSO) except for aqueous extracts that were dissolved in sterile distilled water. The test organisms (*Staphylococcus aureus* and *Klebsiella pneumoniae*) were cultured in MH broth for 24 h at 37 °C. Neomycin was used as the positive control whereas broth (negative), 10% DMSO, and water (solvent controls) were used as negative and solvent controls, respectively. For antibacterial testing against *Staphylococcus aureus* and *Klebsiella pneumoniae*, one hundred microlitres of water were added in each well of the 96 well microtitre. One hundred microlitres of solvent controls and test samples were added to the first wells of the column at an initial concentration of 100 mg/ml of extracts and 50 mg/ml for the positive control and then two-fold serially diluted down the wells. The optical density of *Staphylococcus aureus* and *Klebsiella pneumoniae* was adjusted with MH broth to match that of McFarland standard, equivalent to $10^8$ colony forming units/ml (CFU/ml) at 600 nm. One hundred microlitres of the diluted culture were added to all the wells. The microtitre plates were then incubated for 24 h at 37 °C. After incubation, 40 μl of p-iodonitrotetrazolium violet (INT) indicator was added to evaluate growth inhibition. The results were observed following 30 min incubation at 37 °C. The lowest concentration containing clear wells were considered as the MIC values. The assay was repeated twice with two replicates per assay.

2.5.6. Antimycobacterial activity using the resazurin microplate assay

The resazurin microplate assay (REMA) and broth microdilution method as described by JADAUN et al. (2007) modified by GREEN et al. (2010), to determine the antimycobacterial activity of the extracts, were performed against *Mycobacterium tuberculosis* H37Ra and *Mycobacterium aurum* A+. *Mycobacterium tuberculosis* H37Ra was subcultured in Middlebrook 7H9 broth supplemented with 10% OADC and 0.2% glycerol, and then incubated at 37 °C for 4 weeks in 5% carbon dioxide until logarithmic growth was
reached. Turbidity equivalent to that of McFarland’s No. 1 standard solution for *Mycobacterium tuberculosis* H37Ra was achieved by mixing the culture with a sufficient volume of sterile supplemented Middlebrook 7H9 broth. The test inoculum was obtained by further dilution (1:20) of the suspension with the same culture medium to approximately 6×10⁶ CFU/ml immediately prior to use. *Mycobacterium aurum* A+ was subcultured in Middlebrook 7H9 broth supplemented with 10% OADC and 0.5% glycerol, and incubated for 72 h at 37 °C. The optical density of *Mycobacterium aurum* A+ was adjusted to 0.125 at 550 nm using Middlebrook 7H9 broth enriched with 0.5% glycerol and 10% OADC growth supplement. Rifampicin and streptomycin were used as positive controls whereas broth (negative), 10% DMSO and water (solvent) were used as negative and solvent controls, respectively. The extracts were dissolved in 10% DMSO to a concentration of 100 mg/ml and maintained at room temperature for 1 h to assure their sterilisation. For antimycobacterial testing, 100 µl of Middlebrook 7H9 broth supplemented with 0.5% glycerol, and 10% OADC were then added in each well. Solvent controls and test samples (100 µl) were added to the first wells of the column starting with a concentration of 100 mg/ml of extracts and 50 mg/ml for the positive control and then two-fold serially diluted down the wells. One hundred microlitres of the adjusted bacterial cultures were added to all the wells. The organic and aqueous extracts from each plant were assayed in duplicates. Each microplate with *Mycobacterium tuberculosis* H37Ra as a test organism was incubated for 5 days at 37 °C in a 5% carbon dioxide atmosphere, while the microplates with *Mycobacterium aurum* A+ were incubated for 72 h at 37 °C. After incubation, 32 µl of freshly prepared filter sterilised 0.002% resazurin solution was added to each growth control well only. For *Mycobacterium tuberculosis* H37Ra, the microplates were incubated again at 37 °C in a 5% carbon dioxide atmosphere for 24 h. When a color shift from blue to pink was observed in the growth control sample, 32 µl of resazurin solution was added to each of the remaining wells, and the microplate was further incubated for 24 h. For *Mycobacterium aurum*, the results were observed after 30 min of incubation after adding the indicator. A well-defined pink colour was interpreted as positive bacterial growth, whereas a blue colour indicated the absence of growth.

2.6. Results and discussions
Plants selected in this study are all used in South African TM by various South African tribes for the treatment of tuberculosis and related symptoms. Ten plants were selected based on the available literature of medicinal plants used. The species name, family, traditional uses, voucher specimen numbers, parts used and pharmacological activities of the plants used in the study are given in Table 2.1.
<table>
<thead>
<tr>
<th>Family, species</th>
<th>Voucher specimen No.</th>
<th>Traditional use</th>
<th>Parts used</th>
<th>Previously screened biological activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaryllidaceae</td>
<td>BALUNGI 31</td>
<td>Coughs and colds</td>
<td>Bulbs</td>
<td>Mutagenic and antimutagenic activities of bulbs.</td>
<td>HUTCHINGS et al., 1996; VERSCHAEVE and VAN STADEN, 2008</td>
</tr>
<tr>
<td><em>Brunsvigia grandiflora</em> Lindl.</td>
<td></td>
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</tr>
<tr>
<td>Asparagaceae</td>
<td>BALUNGI 36</td>
<td>Tuberculosis</td>
<td>Shoots</td>
<td>Analgesic and anti-inflammatory activities of roots. Two antiprotzoal compounds were isolated.</td>
<td>HUTCHINGS et al., 1996; VAN WYK et al., 1997; OKETCH-RABAH et al., 1997; HASSAN et al., 2008</td>
</tr>
<tr>
<td><em>Asparagus africanus</em> Lam.</td>
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<tr>
<td>Family, species</td>
<td>Voucher specimen No.</td>
<td>Traditional use</td>
<td>Parts used</td>
<td>Previously screened biological activity</td>
<td>References</td>
</tr>
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</tr>
<tr>
<td>Asparagaceae</td>
<td>BALUNGI 44</td>
<td>Tuberculosis</td>
<td>Leaves, roots</td>
<td>Caryophyllene typesesquiterpene lactone isolated from the leaves was found to have remarkable aspfalcolide effect on the proliferation, migration and tube formation of human umbilical vein endothelial cells. Antibacterial activity of the roots</td>
<td>MABOGO, 1990; STEENKAMP et al., 2007; GHALIB et al., 2012</td>
</tr>
<tr>
<td>Asparagus falcatus (L.) Oberm.</td>
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<tr>
<td>Fabaceae</td>
<td>BALUNGI 43</td>
<td>Tuberculosis, bronchitis, whooping cough, chest complaints and asthma</td>
<td>Leaves, roots</td>
<td>Sedative effects of roots. Antibacterial and cytotoxicity activity of seeds. Five compounds were isolated and identified as isoflavan, quinones and hydroquinones.</td>
<td>HUTCHINGS et al., 1996; ZORE et al. 2007; SIVAKUMAR and ALAGESABOOPATHI, 2008; BOLOU et al., 2011; HATA et al., 2012</td>
</tr>
<tr>
<td>Abrus precatorius subsp. africanus Verdc.</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family, species</td>
<td>Voucher specimen No.</td>
<td>Traditional use</td>
<td>Parts used</td>
<td>Previously screened biological activity</td>
<td>References</td>
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<tr>
<td>Combretaceae</td>
<td>BALUNGI 37</td>
<td>Tuberculosis</td>
<td>Roots</td>
<td>Antibacterial and antifungal activities of the leaves.</td>
<td>SHAI et al., 2008; MABOGO, 1990</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> Engl. &amp; Diels</td>
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<td></td>
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<tr>
<td>Leguminosae</td>
<td>BALUNGI 41</td>
<td>Tuberculosis</td>
<td>Roots</td>
<td>Antibacterial activity of the leaves.</td>
<td>MABOGO, 1990; TOMANI et al., 2008</td>
</tr>
<tr>
<td><em>Indigofera arrecta</em> Benth. ex Harv. &amp; Sond.</td>
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<tr>
<td>Moraceae</td>
<td>BALUNGI 39</td>
<td>Tuberculosis/ ulceration of the lung.</td>
<td>Bark, roots</td>
<td>Antimarial and antibacterial activities of the leaves.</td>
<td>HUTCHINGS et al., 1996; VAN WYK et al., 1997; ELDEEN et al., 2005b; MUREGI et al., 2007</td>
</tr>
<tr>
<td><em>Ficus sur</em> Forssk.</td>
<td></td>
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</tr>
<tr>
<td>Family, species</td>
<td>Voucher specimen No.</td>
<td>Traditional use</td>
<td>Parts used</td>
<td>Previously screened biological activity</td>
<td>References</td>
</tr>
<tr>
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</tr>
<tr>
<td>Polygalaceae</td>
<td>BALUNGI 35</td>
<td>Tuberculosis, blood purification, intestinal sores, sinusitis and gonorrhoea</td>
<td>Whole plant</td>
<td>Antibacterial activity against <em>Gardnerella vaginalis</em> and toxicity.</td>
<td>McGAW et al., 2008a; VAN WYK et al., 1997; VAN VUUREN and NAIDOO, 2010; MUKINDA and EAGLES, 2010</td>
</tr>
<tr>
<td>Polygala fruticosa</td>
<td>P.J. Bergius BALUNGI 40 and 42</td>
<td>Tuberculosis, chest pain, fever and toothache</td>
<td>Roots</td>
<td>Antibacterial activity. Antiviral activity of leaves and roots.</td>
<td>HUTCHINGS et al., 1996; VAN WYK et al., 1997; YFF et al., 2002</td>
</tr>
<tr>
<td>Rubiaceae</td>
<td>BALUNGI 40 and 42</td>
<td>Colds, coughs, bronchitis, asthma, tuberculosis, high blood pressure and jaundice</td>
<td>Leaves, stems</td>
<td>Anti-inflammatory activity.</td>
<td>McGAW et al., 2008a; JÄGER et al., 1996</td>
</tr>
</tbody>
</table>
2.6.1. Plant extraction

In the preparation of plant formulations for pharmacological analysis, extraction is one of the most important steps, as it is necessary to extract the desired plant constituents (AZMIR et al., 2013). Inappropriate extraction methods may result in natural product degradation. The percentage yield of the extracts used in this study are presented in Table 2.2. Most scientists working on secondary metabolites chemistry use dried plant material for many reasons, and one of them is that there are fewer problems associated with large extraction of dried plant material than with fresh ones. Additionally, dried plant material is used by many scientists for extraction because, many if not most plants are used by traditional healers in dried form (ELOFF, 1998b). Hence the use of dried plant materials for preparation of crude extracts in this study.

Plants produce a wide range of secondary metabolites with different functional groups and polarities. The extraction process of bioactive substances from raw plant material is an important stage in the techniques used in natural medicinal preparations. Numerous improvements have been put forward in order to perfect the extraction process (IVANOV et al., 2004). In this study, plant material was ground into powders to increase the surface area for maximum diffusion of the extractable compounds. The plant material was sonicated so as to increase the yield of bioactive compounds while reducing extraction time. Additionally, ice was added during sonication to lower the temperatures, thus preventing decomposition of plant compounds that are sensitive to high temperatures (ELOFF, 1998b).

Although water is used as an extractant in many traditional procedures, organic solvents of varying polarities are generally selected in modern methods of extraction to exploit various solubilities of plant constituents (SARKER et al., 2005). Four solvents (PE, DCM, 80% EtOH and water) were used in this study because of their wide polarity range. The highest quantity of extract yield was observed from the EtOH extract of Polygala fruticosa, whilst the lowest was from PE extracts of the roots of Terminalia phanerophlebia (Table 2.2.). Generally, EtOH was the best extractant giving the highest mass of extracts while PE yielded the lowest mass. The masses of all water extracts were second highest to EtOH for most of the species extracted. When extracting metabolites to screen plants for antimicrobial activity, the effect of the extractant on the subsequent separation is not important, but rather the
solvent used should not interfere with bacterial strains in the bioassay (ELOFF, 1998b; NCUBE et al., 2008). Nonetheless, when extracting plant material intending to isolate chemical constituents only without using a bioassay, solvent toxicity is not important as it can be removed before subsequent isolation procedures.
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant part</th>
<th>Extract</th>
<th>Extract yield % (DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abrus precatorius</em> subsp. <em>africanus</em></td>
<td>Leaves</td>
<td>PE</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCM</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80% EtOH</td>
<td>3.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>2.66</td>
</tr>
<tr>
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<td>Seeds</td>
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<td>80% EtOH</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>2.43</td>
</tr>
<tr>
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<td>PE</td>
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<tr>
<td></td>
<td></td>
<td>DCM</td>
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<td></td>
<td></td>
<td>80% EtOH</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>6.80</td>
</tr>
<tr>
<td>Plant species</td>
<td>Plant part</td>
<td>Extract</td>
<td>Extract yield % (DW)</td>
</tr>
<tr>
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<td>---------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Asparagus falcatus</td>
<td>Leaves</td>
<td>PE</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCM</td>
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</tr>
<tr>
<td></td>
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<td>80% EtOH</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
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<td>Water</td>
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</tr>
<tr>
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</tr>
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<td>Ficus sur</td>
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<td></td>
<td>DCM</td>
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</tr>
<tr>
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<td>80% EtOH</td>
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</tr>
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<td></td>
<td></td>
<td>Water</td>
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</tr>
<tr>
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<td>Plant part</td>
<td>Extract</td>
<td>Extract yield % (DW)</td>
</tr>
<tr>
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<tr>
<td><em>Ficus sur</em></td>
<td>Roots</td>
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<td></td>
<td>Water</td>
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<td>DCM</td>
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<tr>
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<td>80% EtOH</td>
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</tr>
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<td>Water</td>
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<td>Leaves</td>
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</tr>
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<td>Water</td>
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<td>Plant part</td>
<td>Extract</td>
<td>Extract yield % (DW)</td>
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<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
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</tr>
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<td>80% EtOH</td>
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</tr>
<tr>
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<td></td>
<td>Water</td>
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<tr>
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</tr>
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</tr>
<tr>
<td><em>Polygala fruticosa</em></td>
<td>Whole plant</td>
<td>PE</td>
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<td>Extract</td>
<td>Extract yield % (DW)</td>
</tr>
<tr>
<td>------------------------</td>
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</tr>
<tr>
<td><em>Terminalia phanerophlebia</em></td>
<td>Leaves</td>
<td>PE</td>
<td>0.50</td>
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</tr>
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</tr>
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</tr>
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</table>

2.6.2. Antimicrobial and antimycobacterial assay

The MIC values of 68 extracts from 10 plants used in this study are presented in Table 2.3. Analysis of the antimicrobial efficacy of the plant samples was based on the following criteria: MIC values of less than 1.00 mg/ml were considered as having good activity (YORK et al., 2012). Out of 68 extracts tested from different parts of 10 species, 18 were found to have good antimicrobial activity at least against one or more of the tested bacterial strains. Twelve extracts showed good activity against Mycobacterium aurum A+, 11 against Staphylococcus aureus and eight against Mycobacterium tuberculosis H37Ra. It was observed that Mycobacterium aurum A+ was the most susceptible bacterium while Klebsiella pneumoniae was the most tolerant with only six extracts showing good activity against the bacterium. Due to lipopolysaccharides present on their outer membrane, Gram-negative bacteria are usually impermeable to most antibacterial compounds (FENNELL et al., 2004; VORAVUTHIKUNCHAI et al., 2004). This can explain the low number of active extracts against Klebsiella pneumoniae. The water extract of Terminalia phanerophlebia (leaf) showed the best activity with the lowest MIC value of 0.098 mg/ml among the extracts tested. Among the water extracts, Terminalia phanerophlebia leaves exhibited good activity against all tested bacterial strains, with MIC values ranging from 0.098-0.39 mg/ml. The water extracts of Indigofera arrecta (leaf), Pentanisia prunelloides (leaf), and Terminalia phanerophlebia (twig and root) also showed good activity against at least one bacterial strain with MIC values ranging from 0.098-0.78 mg/ml. This was encouraging as many reports often states that water extracts lack bioactivity (MULAUDZI et al., 2009). The evaluation of water extracts aims to mimic the traditional use; therefore, the observed antibacterial activity exhibited by water extracts is of great interest.

Comparing the root and leaf extracts, the leaf EtOH extracts of Indigofera arrecta, showed good antibacterial activity against three bacterial species with MIC values ranging from 0.39-0.78 mg/ml and generally poor for roots. For Pentanisia prunelloides, the root EtOH extracts showed good activity against all bacterial strains tested, with MIC values of between 0.39-0.78 mg/ml. Additionally, the EtOH extracts from the leaves of Pentanisia prunelloides showed good activity against both Mycobacterium aurum and Staphylococcus aureus at MIC values of 0.39 and 0.195 mg/ml respectively. This is noteworthy as it indicates the presence of bioactive compounds in these plants that might be a potential cure for ailments related to
respiratory infection. The palmitic acid previously isolated from roots of *Pentanisia prunelloides* by YFF et al. (2002) may be responsible for the antimicrobial activity of this plant observed in this study. YFF et al. (2002) also did an antiviral test on the root decoction of *Pentanisia prunelloides* and reported an inhibition of *Influenza A virus*. These results make this plant a potentially effective remedy against respiratory diseases, and the evaluation of palmitic acid against respiratory microbes is required.

For *Terminalia phanerophlebia* leaf, root, and twig extracts, the EtOH extracts showed good activity against the majority of the bacterial strains tested, with MIC values ranging from 0.195 to 0.78 mg/ml. This, however, was excluding the leaf extract that did not exhibit good activity against one strain and root extract that also did not show good activity against two strains. In a study on antimicrobial properties done by SHAI et al. (2008) against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*, the leaf extracts of *Terminalia phanerophlebia* exhibited good antimicrobial activity which was also the case in this study against the tested bacteria. In addition, through a literature investigation conducted by NAIR et al. (2012), several alkaloids were reported to have been isolated from the stem of *Terminalia phanerophlebia* which exhibited cyclooxygenase-2 enzyme activity. The antimicrobial activity observed from different parts of this plant might be due to those sterols (cholestane triterpenoids: β-sitostenone, stigmast-4-ene-3,6-dione, and β-sitosterol). It is, therefore, important to evaluate these compounds in bioassays against these microorganisms. *Terminalia* plants were confirmed to have medicinal importance when *Terminalia sericea* Burch. ex DC. was included in the African herbal pharmacopeia representing the top 50 African medicinal plants (ELOFF et al., 2008). The leaves and bark of *Terminalia phanerophlebia* were reported by SIBANDZE (2009) to possess high flavonoid and phenolic content. Flavonoids are known to have antimicrobial, anti-inflammatory, anti-cancer and antiviral activities (HAVSTEEN, 2002). Reports on antmycobacterial activities of flavonoids through inhibiting enzymes involved in biosynthesis of mycolic and fatty acid have been made (KUETE et al., 2010a). Additionally, flavonoids have been observed to possess the same modulatory properties of isoniazid (INH) and hence could be taken with anti-tuberculosis treatment for preventing or counteracting INH resistance (MACABEO et al., 2012). The genus *Terminalia* has been reported to have antmycobacterial properties and this was evident in a study done by KUETE et al. (2010b) where compounds isolated from *Terminalia superba* Engl. & Diels inhibited *Mycobacterium* species. Good antimicrobial activity is sometimes associated with toxicity, however, the leaf
extracts of *Terminalia phanerophlebia* demonstrated non-toxicity in a study done by SIBANDZE (2009). The interaction or coinfection of tuberculosis microorganisms and malarial parasites is of importance. Of particular interest is a study by SIBANDZE (2009) which confirmed the activity of *Terminalia phanerophlebia* as an antimalarial agent and this current study has demonstrated the same plant as an anti-tuberculosis agent.

For *Ficus sur* (bark and root), only the EtOH extract (root) showed good activity with an MIC value of 0.78 mg/ml against *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*, as well as 0.195 mg/ml against *Staphylococcus aureus*. From a previous study, good antibacterial activities were observed on tested extracts of the leaves, roots and bark of *Ficus sur* (ELDEEN et al., 2005b). The activity of this plant could be due to the triterpene compounds found in the latex isolated in a previous study by FELEKE and BREHANE (2005), which also warrants testing in similar bioassays.

For *Leonotis intermedia* (leaf and stem), only the EtOH extract (leaf) showed good activity with MIC value of 0.195 mg/ml against *Mycobacterium aurum* A+ and 0.78 mg/ml against *Staphylococcus aureus*. These findings are very interesting as they indicate the antimicrobial activity of this plant. Of all the extracts of *Asparagus africanus* and *Asparagus falcatus*, good activity was observed only in the EtOH extracts with both having an MIC value of 0.39 mg/ml against *Mycobacterium aurum* A+. *Asparagus* species are known to have steroidal saponins as their major bioactive constituents; therefore, the antimycobacterial activity of these two species could be due to these groups of compounds (NEGI et al., 2010).

For *Abrus precatorius* subsp. *africanus*, the leaves showed good antimycobacterial activity against both *Mycobacterium* species tested, with MIC values of 0.195 and 0.78 mg/ml, respectively. The leaf is one of the plant parts reported to be used for tuberculosis treatment in South Africa, thus the antimycobacterial activity exhibited by the leaf EtOH extracts was noteworthy. Additionally, good antibacterial activity was also exhibited against *M. aurum* by the leaf DCM and seed EtOH extracts of *Abrus precatorius* subsp. *africanus*, with MIC value of 0.78 mg/ml. The stem EtOH extracts of *Abrus precatorius* subps. *africanus* was found to have partial activity against *Mycobacterium tuberculosis* in a study done by ANTOUN et al. (2001). Phytochemical research done in a previous study by TAUR and PATIL (2011) from the root and aerial parts of this plant showed the presence of triterpenoids and saponins.
Saponins are known to have a broad spectrum of pharmacological activities, including antimicrobial property (SPARG et al., 2004).

In view of the fact that these plants were selected based on their traditional uses against tuberculosis and related symptoms, these findings are noteworthy. The antimicrobial activity observed from the extracts of *Abrus precatorius* subsp. *africanus*, *Asparagus africanus*, *Asparagus falcatus*, *Ficus sur*, *Indigofera arrecta*, *Leonotis intermedia*, *Pentanisia prunelloides* and *Terminalia phanerophlebia* advocates that these plants possibly have antimycobacterial compounds. Therefore, further studies aimed at isolating bioactive compounds of these plants are necessary as the observed findings substantiate that they might comprise alternative future candidates for tuberculosis treatment. All the extracts of *Brunsvigia grandiflora* and *Polygala fruticosa* P.J. Bergius did not display good activity against all the screened bacterial strains in these assays despite being reported to be used in the treatment of tuberculosis and related symptoms. However, bioactivity cannot be completely ruled out from such plant species as they could be active against other bacterial strains that cause tuberculosis and related respiratory ailments. The other possible explanation could be that antimicrobial effects of these plants are not mediated through direct inhibition on microbial growth but rather through immunostimulation or the compounds potentially active require metabolic activation by certain enzyme(s) *in vivo*. 
Table 2.3: Antibacterial (MIC values) effects of plants used traditionally as remedies in the treatment of tuberculosis and related symptoms in South Africa

<table>
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<th>Plant part</th>
<th>Extract</th>
<th>Antibacterial activity</th>
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L: leaves, R: roots, B: bark, Blb: bulb, S: seeds, Stm: stem, Wp: whole plant, T: twigs, MIC: minimum inhibitory concentration, DCM: dichloromethane, PE: petroleum ether, EtOH: ethanol, the values highlighted in bold are considered very active, Neomycin, Rifampicin and Streptomycin are positive controls, -: not determined. The concentration of plant extracts was 100 mg/ml.
2.7. Conclusions

Two extracts of *Abrus precatorius* subsp. *africanus*, *Terminalia phanerophlebia*, *Pentanisia prunelloides*, *Indigofera arrecta* and one extract of *Asparagus falcatus*, *Asparagus africanus*, *Leonotis intermedia*, as well as *Ficus sur* showed good antimicrobial activity against at least one of the bacterial strains tested. The results indicate that some of the plants such as *Abrus precatorius* subsp. *africanus*, *Asparagus africanus*, *Asparagus falcatus*, *Indigofera arrecta*, *Leonotis intermedia*, *Ficus sur*, *Pentanisia prunelloides* and *Terminalia phanerophlebia* have antimycobacterial compounds. Significant *in vitro* antimycobacterial activities have been demonstrated by extracts of South African plant species from different families and genera in this study. This provides supporting evidence that South Africa has diverse flora which has a potential for the discovery of metabolites that are active against *Mycobacterium tuberculosis*. Additionally, further phytochemical and pharmacological studies (such as isolation of active compounds and antimycobacterial evaluation against resistant tuberculosis strains) of these plants are worthwhile as they form a good preliminary basis for the selection of candidate plants. The fact that some species of the tested plants such as *Brunsvigia grandiflora* and *Polygala fruticosa* did not display good antimicrobial activity does not mean that they may be inactive *in vivo*; thus, it should be noted that they only demonstrated weak activity *in vitro*. Weak activity might mean that the plant species are used to treat the symptoms of various respiratory ailments rather than the disease itself.
Chapter 3: Inhibition of cyclooxygenase enzyme as an evaluation of anti-inflammatory property of selected plants

3.1. Introduction

In order for humans to survive, they have to be able to eliminate foreign invaders such as agents of infection and damaged tissues from their system. The elimination of foreign material is mediated by a complex response called inflammation. Inflammation is defined as a protective reaction evoked by body cells or tissues in response to various factors such as bacterial infections, allergic reactions and chemical injury (IWALEWA et al., 2007; KUMAR et al., 2013). The inflammation response is an important function of the immune system that is required to protect the host against microorganisms and initiate specific immunity (SHIN et al., 2010). Inflammation aids in eliminating infections and other harmful stimuli and initiates healing. However, the response and the subsequent healing process can themselves cause substantial damage (KUMAR et al., 2013). The mechanisms of the inflammatory response that destroy and eradicate microorganisms and dead tissues are also capable of injuring normal tissues (KUMAR et al., 2013; KATARIA and KAUR, 2013). Thus, injury may accompany entirely normal, beneficial inflammatory reactions, and the damage may even become the dominant feature when the infection is severe. Some of the most complicated human diseases are disorders that result from inappropriate, often chronic, inflammation.

Inflammation can be acute or chronic and can be beneficial or harmful (SUGAWARA, 2009). Acute inflammation is the initial response of the immune system to harmful stimuli (VASSILEVA and PIQUETTE-MILLER, 2010). The acute inflammatory response is characterised by exudation of fluids and plasma proteins as well as movement of leukocytes (neutrophils) into the harmed area (IWALEWA et al., 2007). Acute inflammation is believed to be helpful when it occurs under normal circumstances as it results in healing and serves as a defence mechanism against bacteria, viruses and parasites (VASSILEVA and PIQUETTE-MILLER, 2010). When the body is unable to destroy the invading foreign object, chronic inflammation occurs (VASSILEVA and PIQUETTE-MILLER, 2010). Chronic inflammation is characterised by mononuclear immune cell infiltration, simultaneous
tissue destruction and healing attempts which include angiogenesis and fibrosis. Chronic inflammation is evident in the case of persistent infections like tuberculosis, rheumatoid arthritis, cancer and chronic lung diseases (KATARIA and KAUR, 2013).

Inflammatory responses are major contributors to diseases and disorders as its symptoms appear when the body recognizes the injury and repairs the damage (IWALEWA et al., 2007). Without inflammation, wounds would never heal and infections would go unchecked. The symptoms of an inflammation response are redness, pain, and swelling with impaired functioning of the inflamed area. Redness of the inflamed area is due to an increased quantity of blood so that the smallest capillaries are distended with red particles (VASSILEVA and PIQUETTE-MILLER, 2010).

Inflammation treatment usually consists of the use of aspirin and similar anti-inflammatory drugs (GALE et al., 2007). However, currently used anti-inflammatory drugs have serious side effects. Worldwide, researchers continue the search for new anti-inflammatory drugs and there is growing interest by the pharmaceutical and herbal industries in the potential of plants as anti-inflammatory agents (GALE et al., 2007). As a result of scientific research taking place in various countries at present, a number of anti-inflammatory compounds have been isolated from medicinal plants. Thus, evaluating medicinal plants that are used traditionally in the treatment of pain related infections in search for potent anti-inflammatory agents with reduced or no side effect is proving to be worthwhile.

3.2. Overview of inflammation response

The inflammation response is controlled by a number of chemical mediators. Upon pathogen invasion, mammalian monocytes or macrophages identify lipopolysaccharides (LPS) of bacteria through transmembrane signaling Toll-like receptor-4 (TLR-4), thus signalling commencement of normal host defence mechanisms (SHIN et al., 2010). The chemical mediators are released as plasma proteins or come from mast cells, platelets, neutrophils and monocytes or macrophages (SHIN et al., 2010). The inflammation process mediators are termed pro-inflammatory fundamental factors, and they result in inflammations of different severity depending on the duration of the injury (IWALEWA et al., 2007). The chemical mediators include nitric oxide, prostaglandins, leukotrienes, vasoactive amines and cytokines.
(VANE and BOTTING, 1987). These chemical mediators bind to target receptors on the cells thereby increasing vascular permeability, promoting neutrophil chemotaxis, stimulating smooth muscle contraction, increasing direct enzymatic activity, inducing pain and mediating oxidative damage in the process (IWALEWA et al., 2007).

The complex inflammatory response begins with the release of arachidonic acid (AA), a 20 carbon tetraenoic fatty acid, from the plasma membrane phospholipids following stimulation (SIMMONS et al., 2004). Tissue damage activates phospholipase A2 enzyme which cleaves AA from cell membrane phospholipids. Upon release, AA proceeds down either the lipoxygenase (LOX) or cyclooxygenase (COX) pathway leading to the formation of thromboxane or prostaglandins respectively (GARCIA-VALASCO and RIZK, 2010). Cyclooxygenase and LOX pathways are responsible for controlling the biosynthesis of eicosanoids. The COX-derived eicosanoids are prostanoids, like prostaglandin 12 and E2 (PG12 and PGE2) that are involved in platelet aggregation regulation of gastrointestinal and renal blood flow (SIMMONS et al., 2004). Prostaglandin 12 and PGE2 can however be powerful vasodilator agents that play a role in the vascular signs of the inflammation response (WISASTRA and DEKKER, 2014). Prostaglandins play a vital role in the homeostasis of several tissues and organ system, including the gastrointestinal tract, renal, pulmonary, cardiovascular, and the reproductive system (GALE et al., 2007). They also play a crucial role in the regulation of blood pressure and coagulation. Prostaglandins are produced by most human cells and their overproduction is the main cause of inflammation. Cyclooxygenase enzyme/prostaglandin H synthase or LOX are the major enzymes involved in the conversion of AA to prostaglandin (SIMMONS et al., 2004). During inflammation processes, both leukotrienes and prostanoids are synthesised and released by cells in concentrations sufficient to affect their biological processes such as vascular changes, increase in body temperature, and migration of leukocytes.

3.3. Cyclooxygenase enzymes

Cyclooxygenase is the key and rate limiting enzyme that catalyses the first step of prostaglandin synthesis, which is the main cause of inflammation (VERNIERI et al., 2013). There are three forms of COX enzymes that are known to convert AA to prostanoids; they are COX-1, COX-2 and COX-3 (GILROY et al., 1999; MENDES et al., 2012). All these COX
enzymes are named according to the order in which they were discovered. Cyclooxygenase enzymes were initially believed to be expressed constitutively with constant levels in individual tissues (PORCHER et al., 2002). However, the discovery of COX-2 between 1989 and 1992 gave rise to the concept of constitutive COX-1 and inducible COX-2 (SIMMONS et al., 2004).

Cyclooxygenase-1 and COX-2 are similar in many respects, however there are significant differences that distinguishes the two isoenzymes. Cyclooxygenase-1 is a membrane bound protein that resides in the endoplasmic reticulum of prostanoid forming cells (MBONYE et al., 2006). The amino acids in the structure of COX-1 form a long narrow channel that ends with a hairpin-like bend at the end. The mRNA of this enzyme is about 4.5 Kb (POPOVICI et al., 2011). Cyclooxygenase-1 is the first isoenzyme that is expressed constitutively in resting cells of most tissues and shares 60% homology at the amino acid level with COX-2 enzyme (PATRIGNANI et al., 2005). This first isoenzyme functions as a housekeeping enzyme and is responsible for maintaining homeostasis (gastric and renal integrity) as well as normal production of prostaglandins (VERNIERI et al., 2013). This enzyme has a molecular weight of 71 Kda.

Cyclooxygenase-2 enzyme is about 82 Kb long and is located on chromosome 1. It consists of 10 exons and 9 introns. The mRNA of COX-2 is about 2.8 Kb. Cyclooxygenase-2 is mainly located in the lumen of the endoplasmic reticulum where the oxidising conditions favor its proper dimerisation (POPOVICI et al., 2011). Just like COX-1, COX-2 has a molecular weight of 71 Kda. It is an “inducible enzyme”, upregulated by infection and is responsible for the inflammatory response. The constitutive presence of COX-2 has been recently highlighted in the central nervous system, kidney, and endothelial cells, but is virtually absent in most other tissues (VERNIERI et al., 2013). The expression of COX-2 is significantly upregulated in neoplastic tissues and as part of various acute as well as chronic inflammatory conditions. The stimulators of COX-2 expression are epidermal growth factors, platelet derived growth factors, pro-inflammatory cytokines, tumor promoters, bile acids and ultraviolet B irradiation (POPOVICI et al., 2011). Cyclooxygenase-2 enzyme is the main target for treatment of inflammatory diseases.

Cyclooxygenase-3 enzyme is a splice variant of COX-1 (PATRIGNANI et al., 2005). Although COX-3 enzyme is made from the COX-1 gene, it retains intron 1 in its mRNA and
was reported to be expressed in the canine cerebral cortex (PATRIGNANI et al., 2005). Cyclooxygenase-3 is pharmacologically different to COX-1 and COX-2 even though it is derived from COX-1. This enzyme is less potent and produces less prostaglandin than either COX-1 or COX-2. However, research has revealed that COX-3 might have a role in remission periods in chronic inflammatory diseases and could be involved in ovarian, colonic and cervical cancer (POPOVICI et al., 2011).

3.4. Inflammation and tuberculosis

One of the symptoms of tuberculosis is chest pain caused by inflammation of the membranes lining the lungs. Several tuberculosis symptoms are associated with the pronounced host inflammatory responses that occur as the immune system attempts to recover (NANDI and BEHAR, 2011). In the case of pulmonary tuberculosis, alveolar macrophages control persistant inflammatory responses in the lungs by producing chemical mediators that leads to granuloma formation (SHINOHARA et al., 2009). The main roles of granulomas formed in the lungs during tuberculosis infection are confinement and containment of bacteria, and the inflammatory response to the bacteria itself (SHINOHARA et al., 2009). Tuberculosis is characterised by inflammation when the disease is latent and active (KAUFMANN and DORHOI, 2013). During latent tuberculosis infection, the immune system holds the pathogen through controlled inflammation, causing minimal collateral damage. The lungs are the entrance route for mycobacteria species, and they allow the bacteria to evade protective host defenses, forming pathological lesions that are essential for transmission of the bacterial strain from the granuloma lung lesion to the alveoli and aerosols (IVANYI and ZUMLA, 2013; NANDI and BEHAR, 2011).

The application of tuberculosis anti-inflammatory treatment is for lessening injurious host inflammation reactions that results in pathological lung lesions (KAUFMANN and DORHOI, 2013). Thus, the target of tuberculosis anti-inflammatory treatment is lung granulomatous lesions. These granulomatous lesions are produced by a variety of immune cells such as monocytes, lymphocytes and neutrophils which produce prostaglandins (IVANYI and ZUMLA, 2013). Therefore, all immune cells that produce granulomatous lesions can be targets for action of COX inhibitory drugs (IVANYI and ZUMLA, 2013).
3.5. Anti-inflammatory agents

Anti-inflammation refers to the ability of a substance to alleviate pain by blocking production of prostaglandins. There are two types of anti-inflammatory drugs, and they include steroidal/or glucocorticoid and non-steroidal anti-inflammatory drugs (NSAIDs) (DINARELLO, 2010). Glucocorticoids are used on a wide scale for treatment of several inflammatory diseases and they have a unique ability of inhibiting both COX and LOX pathways simultaneously (UMARU et al., 2009; DINARELLO, 2010). They inhibit COX-2 by down regulating its transcription. Non-steroidal anti-inflammatory drugs are compounds of non-steroidal origin that are capable of reducing or inhibiting tissue injury associated with inflammatory responses, and they include aspirin, and several other selective and non-selective inhibitors of COX (UMARU et al., 2009). The main benefits of NSAIDs are analgesic, antipyretic and anti-inflammatory effects (AWOFISAYO et al., 2008). These drugs are generally used for relief of symptoms of rheumatoid arthritis, osteoarthritis, dysmenorrhea, pyrexia and post-operative pain (DINARELLO, 2010). Due to the acidic nature of NSAIDs, they are easily absorbed in the stomach and intestinal mucosa. Non-steroidal anti-inflammatory drugs NSAIDs are mobilised in the liver through oxidation and conjugation to inactivate metabolites and are excreted partially in bile or urine (AWOFISAYO et al., 2008).

However, both NSAIDs and glucocorticoids have the potential to interfere with the healing process as they inhibit inflammation (WRIGHT, 2002). Glucocorticoids such as prednisone, prednisolone, and hydrocortisone which are naturally secreted by the adrenal glands or chemically synthesised are associated with side effects such as retarded growth in children, immunosuppression, muscle breakdown, increased blood pressure and glaucoma. The use of NSAIDs is associated with a number of adverse effects (GALE et al., 2007). These include kidney damage, worsening asthma, cardiovascular ailments, and damage to the gastrointestinal tract (DINARELLO, 2010). Non-steroidal anti-inflammatory drugs have injurious effects to the upper and lower gut that occur by depleting COX-1-derived prostaglandin-causing topical injury to the mucosa. These NSAIDs may be associated with gastrointestinal ailments ranging from mild to severe dyspeptic symptoms, development of gastric or duodenal ulceration, haemorrhage or perforation and other events which may lead to hospitalisation or worse, death.
As a result of the association of NSAIDs with adverse effects, as an alternative, more selective agents of COX were developed. Selective COX-2 inhibitors were introduced in the 1990s as first line drugs for osteoarthritis and rheumatoid arthritic treatment (DINARELLO, 2010). This class of drugs includes the widely used rofecoxib, etoricoxib and celecoxib (WRIGHT, 2002). The advantages of selective COX-2 inhibitors were in their potent anti-inflammatory action and lower risk of gastrointestinal problems as they leave COX-1 gastroprotective prostaglandin unopposed (SOLHEIM et al., 2013). In high risk cancer patients, the adenocarcinoma cells overexpress COX-2, and selective COX inhibitors play a beneficial role by reducing colon cancer development (SOLHEIM et al., 2013). However, a possible link between selective COX-2 inhibitors and increased cardiovascular as well as cerebrovascular risk has been suggested (WRIGHT, 2002). Recently there have been concerns regarding selective COX-2 inhibitors, as they are believed to contribute to the onset of acute myocardial infarction and thromboembolic events (HOWARD and DELAFONTAINE, 2004; SEGEV and KATZ, 2004; UMARU et al., 2009). Thus, there is still a need to develop more effective and safer COX inhibitors.

Due to numerous problems associated with the currently available anti-inflammatory drugs, search has intensified on plants for alternative agents (OKOLI et al., 2003). Various plants have been used in folk medicine for the treatment of inflammatory disorders (AGNIHOTRI et al., 2009). Some of the traditionally used anti-inflammatory plants include Aloe vera (L.) Burm.f., Harpephyllum caffrum Bernh., Helichrysum nudifolium (L.) Less, and Leonotis leonurus (L.) R.Br. and these have demonstrated varying activity in in vitro inflammatory models (TAYLOR et al., 2001; AGNIHOTRI et al., 2009). Worldwide, several scientific studies have shown that plants do possess anti-inflammatory effects (TUNÓN et al., 1995; AGNIHOTRI et al., 2009; OLUKUNLE et al., 2011). Similarly, many South African medicinal plants have been suggested as potential sources of anti-inflammatory agents (JÄGER et al., 1996; McGAW et al., 1997; SHALE et al., 1999). Modern allopathic drugs are single active components that target one specific pathway. Plants contain more than one ingredient that work on targeted elements of the complex cellular pathway (KUMAR et al., 2013). Various anti-inflammatory compounds such as curcumin, pentacyclic triterpenic acid and its derivatives have been structurally and pharmacologically isolated from plants (OKOLI et al., 2003). The main chemical classes of anti-inflammatory agents from plants include compounds such as polyphenols, flavonoids, terpenoids, alkaloids, anthraquinones,
lignans, polysaccharides, saponins and peptides (OKOLI et al., 2003; AGNIHOTRI et al., 2009). Therefore, plants with anti-inflammatory property and which lack adverse effects can be used as therapy in the management of inflammatory conditions.

Plants used for treatment of tuberculosis and related symptoms were selected for this study, to evaluate their anti-inflammatory properties since the tuberculosis disease is associated with inflammation. Pulmonary tuberculosis often leads to lung injury, pathological and inflammatory responses (DAVIES et al., 1997; DEY et al., 2003). Mycobacteria species are known to induce expression and induction of matrix metalloproteinase-9 which is suggested to be involved with COX-2 reliant signaling events (BANSAL et al., 2009). Therefore the development of selective COX-2 inhibitors could be an important step in the therapeutic treatment of inflammatory pulmonary tuberculosis. Thus, the COX-2 enzyme was selected for this study. Additionally, it is important to screen medicinal plant extracts in different in vitro assays, because there is a possibility of losing other potentially useful bioactive compounds when there is evaluation of only a single biological activity. Unlike drugs which are single active components that target one specific illness, herbal medicines work in a way that depends on an symphonic method (KUMAR et al., 2013). Thus, the plants screened for their antimicrobial properties in Chapter 1, were selected for anti-inflammation screening in this Chapter.

3.6. Materials and methods

Preparation of plant extracts and COX-2 bioassay were performed as detailed below.

3.6.1. Preparation of plant extracts

The extracts were prepared as described in Section 2.5.1. The stored dried sample extracts were resuspended in 70% EtOH for organic solvent extracts (PE, DCM and EtOH) and in water for aqueous extracts to a concentration of 10 mg/ml.

3.6.2. Substrate and enzyme preparation
The substrate $^{14}\text{C}$-arachidonic acid (radio labeled AA) was obtained from Amersham (GE Healthcare, UK). Radio-labeled AA (100 µl) were diluted with unlabelled AA (6.75 µl) (Sigma-Aldrich, Germany) to obtain the final concentration (17 Ci/mol, 30 µM) required for the bioassay.

The COX-2 enzyme used in this study was obtained from Sigma-Aldrich (USA). A stock solution of the enzyme was kept at -70 °C until use. The COX enzyme was diluted with Tris(hydromethyl)aminomethane (TRIS) storage buffer (pH 8.0) to obtain 60 µl of 75 units enzyme concentration per aliquot. The prepared COX enzyme (50 µl, 75 units) was kept in an ultra-freezer at -70 °C until required.

### 3.6.3. Cyclooxygenase inhibitory activity

The COX-2 inhibition assay was performed as described by JÄGER et al. (1996) and modified by ZSCHOCKE and VAN STADEN (2000). Prepared stock solution of COX-2 enzyme (60 µl) at -70 °C was activated with 1250 µl of co-factor solution (3 mg/ml epipherine, 6 mg/ml glutathione in 10 ml Tris buffer pH 8.0, and 100 µl hematin solution) and incubated on ice for 5 min. Plant extracts (2.5 µl) were added to 17.5 µl of distilled water, (for water extracts only 20 µl of the extract were added) to give a final concentration of 250 µg/ml of each organic extract per test solution (2 mg/ml for water extract per tester solution) and this was done in duplicates. A similar 200 µM indomethacin was used as a positive control. Solvent blank and background (2.5 µl ethanol and 17.5 µl of water) were used as negative controls. The enzyme solution (60 µl) was added to the test solution and pre-incubated at room temperature for 5 min. The reaction was initiated by adding 20 µl of $^{14}\text{C}$-AA to the test solutions. The enzyme in the background was inactivated by adding 10 µl 2N hydrochloric acid (HCL) before incubating the solution at 37 °C for 10 min. The incubation was stopped (after 10 min) and the reaction was terminated by adding 10 µl of 2N HCL to each test solution. A total of 4 µl of 0.2 mg/ml of unlabelled prostaglandin (PGE$_2$: PGF$_{2\alpha}$ 1:1) from Sigma-Aldrich were added to each Eppendorf as a carrier solution. Silica gel (particle size, 0.63-0.200 mm, Merck) was used to pack silica columns to a height of 3 cm using Pasteur pipettes. To separate the prostaglandins and unmetabolised AA, the test solution was applied to the column with 1 ml of eluent 1 [hexane: 1, 4-dioxan: glacial acetic acid (70:30:0.20 v/v/v)]. The AA was eluted first with 4 ml of eluent 1 and the products of
prostaglandin were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15)] and collected in scintillation vials. A total of 4 ml of scintillation fluid was added to each vial and a scintillation counter (Beckman LS 6000 LL) was used to count the disintegration per minute (DPM) of radioactive material. The assay was performed in replicates and repeated three times. The following formula was used to calculate the percentage of inhibition for the extracts:

\[
\text{COX inhibition (\%)} = \left\{ 1 - \left( \frac{\text{DPM extract} - \text{DPM background}}{\text{DPM solvent blank} - \text{DPM background}} \right) \right\} \times 100
\]

Where DPM extract, DPM background and DPM solvent blank represent the disintegrations per minute for extract, background and solvent blank, respectively.

3.7. Results and discussions

3.7.1. Anti-inflammatory activity

A total of 68 extracts from 10 plants used for the treatment of tuberculosis and related symptoms in South Africa were screened for their ability to inhibit the COX-2 enzyme. Table 3.1 shows the results as percentage inhibition of prostaglandin synthesis by the extracts in the COX-2 assay. For inhibition of the enzyme by extracts, four different levels of activity were defined; inhibition below 20% was considered insignificant, between 20% to 40% low, between 40% to 70% moderate and between 70% and 100% as high (TUNÓN et al., 1995). The highest COX-2 inhibition was exhibited by PE extract from the roots of Pentanisia prunelloides (86.9%). In studies done by YFF et al. (2002) and LINDSEY et al. (1999), the EtOH, ethyl acetate and water extracts of Pentanisia prunelloides (leaves and roots) demonstrated high inhibition of COX enzyme. The roots of Pentanisia prunelloides are reported to be used to relieve chest pain and bacterial infection (YFF et al., 2002). The root EtOH extract of Pentanisia prunelloides exhibited remarkable antimicrobial activity against bacterial strains associated with respiratory ailments (Chapter 2, Table 2.3), and in the present study PE extracts of the same plant part demonstrated high COX-2 inhibition, thus, these findings supports the use of this plant in South African TM for treatment of inflammation associated diseases like pulmonary tuberculosis.
The DCM extracts of *Abrus precatorius* subsp. *africanus* (leaves) and *Ficus sur* (bark) exhibited high COX-2 inhibition with percentages of 77.9 and 73.9, respectively. In previous research by KUO et al. (1995) on potent antiplatelet, anti-inflammatory and antiallergic properties of isoflavanquinones from the roots of *Abrus precatorius* subsp. *africanus*, abruquinones A, B, D and F exhibited remarkable anti-inflammatory effects. Therefore, the noteworthy anti-inflammatory activity exhibited by the extract from *Abrus precatorius* subsp. *africanus* in the present study could be from these abruquinones.

Both the roots and bark of *Ficus sur* are used by the Zulu people of South Africa for traditionally treating pulmonary tuberculosis. These plant parts showed high COX-1 inhibitory activity in a study by ELDEEN et al. (2005b) and low COX-2 inhibition. In this study a different solvent (DCM) from the ones used by ELDEEN et al. (2005b) (ethyl acetate, EtOH and water) showed the bark of *Ficus sur* to have high COX-2 inhibition.

Out of a total of 68 extracts tested, only three exhibited high COX-2 inhibition, 14 moderate, 20 low and the remaining 31 showed insignificant inhibition. Of 17 DCM extracts tested, two exhibited high COX-2 inhibition, five showed moderate and the rest showed low to insignificant activity. For PE extracts, only one showed high COX-2 inhibition, four showed moderate and the rest had low to insignificant activity. For EtOH extracts only *Abrus precatorius* subsp. *africanus* (leaves), *Asparagus africanus* (leaves), *Ficus sur* (roots), and *Leonotis intermedia* showed moderate COX-2 activity with percentage inhibition ranging from 41.4 to 50.6. The other EtOH extracts exhibited low to insignificant activity. The majority of the water extracts showed insignificant to low COX-2 inhibition with the exception of that of *Asparagus africanus* that showed moderate activity (56.6%). In the case of water extracts showing weak or no activity in these assays, it is worth mentioning that high dosages are frequently used in TM (McGAW et al., 1997). According to JÄGER et al. (1996), a species with an anti-inflammatory compound has a potential to be developed into an anti-inflammation product. Cyclooxygenase-2 inhibition is beneficial in clinical conditions as it provides therapeutic effects and its high activity is desirable (BLOBAUM and MARNETT, 2007). However, COX-2 inhibitors are currently regarded as dangerous, as their prolonged use may cause side effects in humans (BLOBAUM and MARNETT, 2007).

All plant species that were evaluated in this study for inhibition of COX-2 are frequently used for the treatment of tuberculosis and related symptoms. The inhibition of the tested
COX enzyme shown by some of the evaluated plants supports their use in South African TM for the treatment of pain-related ailments. Additionally, anti-inflammatory property observed by some of the investigated plant extracts in this study indicates the presence of bioactive agents that warrant further investigation. The extracts that exhibited weak or no activity might be active when concentrations higher than those evaluated are used. Since lipophilic compounds are more extractable by non-polar solvents and have far better resorption through the cell membrane, activity exhibited by polar solvents specifically at low concentrations is noteworthy (ZSCHOCKE and VAN STADEN, 2000). The high COX-2 inhibitory activity exhibited by the DCM leaf extracts of Abrus precatorius subsp. africanus is important for medicinal plant species conservation, as the leaves can be sustainable harvested while using the plants for medicine without threat to their survival. When treating inflammation, targeting the desired area is not easy as the process has many mediators and pathways that can lead to many pathological changes. The results obtained in this study are depended strongly on the test system used, therefore additional systems are needed to evaluate anti-inflammatory activity. Anti-inflammatory drugs with low COX-1 and high COX-2 inhibition properties are required. Therefore, it would be ideal to evaluate further the DCM extract of Abrus precatorius subsp. africanus (leaves) and Ficus sur (bark) as well as the PE extract of Pentanisia prunelloides (roots) for their COX-1, LOX inhibition, and to check if these plants inhibit these other enzymes, because high inhibition of both enzymes (COX-1 and COX-2) is associated with adverse effects.
Table 3.1: Anti-inflammatory activity (COX-2) of extracts from plants that are used for the treatment of tuberculosis and related symptoms in South Africa

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant part used</th>
<th>Percentage inhibition of cyclooxygenase-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PE</td>
</tr>
<tr>
<td>Abrus precatorius subsp. africanus</td>
<td>Leaves</td>
<td>11.9±0</td>
</tr>
<tr>
<td>Abrus precatorius subsp. africanus</td>
<td>Seeds</td>
<td>39.0±0</td>
</tr>
<tr>
<td>Asparagus africanus</td>
<td>Leaves</td>
<td>9.3±0</td>
</tr>
<tr>
<td>Asparagus falcatus</td>
<td>Leaves</td>
<td>9.2±0</td>
</tr>
<tr>
<td>Brunsvigia grandiflora</td>
<td>Bulbs</td>
<td>23.4±0</td>
</tr>
<tr>
<td>Ficus sur</td>
<td>Bark</td>
<td>12.4±0</td>
</tr>
<tr>
<td>Ficus sur</td>
<td>Roots</td>
<td>44.9±0.5</td>
</tr>
<tr>
<td>Indigofera arrecta</td>
<td>Leaves</td>
<td>24.7±2.6</td>
</tr>
<tr>
<td>Indigofera arrecta</td>
<td>Roots</td>
<td>52.2±0</td>
</tr>
<tr>
<td>Leonotis intermedia</td>
<td>Leaves</td>
<td>36.9±0</td>
</tr>
<tr>
<td>Leonotis intermedia</td>
<td>Stems</td>
<td>0.0±0</td>
</tr>
<tr>
<td>Pentanisia prunelloides</td>
<td>Leaves</td>
<td>2.5±0</td>
</tr>
<tr>
<td>Pentanisia prunelloides</td>
<td>Roots</td>
<td>86.9±0</td>
</tr>
<tr>
<td>Polygala fruticosa</td>
<td>Whole plant</td>
<td>54.7±0</td>
</tr>
<tr>
<td>Terminalia phanerophlebia</td>
<td>Leaves</td>
<td>29.0±1.1</td>
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<tr>
<td>Terminalia phanerophlebia</td>
<td>Roots</td>
<td>23.9±0</td>
</tr>
<tr>
<td>Terminalia phanerophlebia</td>
<td>Twigs</td>
<td>67.8±0.05</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>-</td>
<td>79.2</td>
</tr>
</tbody>
</table>

DCM: dichloromethane, PE: petroleum ether, EtOH: ethanol. The concentration of plant extracts was 250 µg/ml.
3.8. Conclusions

The highest inhibition of COX-2 enzyme in this study was shown by the PE extracts of *Pentanisia prunelloides* (roots) at 86.9%. The DCM extracts of *Ficus sur* (bark) and *Abrus precatorius* subsp. *africanus* (leaves) exhibited high inhibition of COX-2 at inhibition percentages of 73.9 and 77.9 respectively. In terms of medicinal plant species conservation, the high COX-2 inhibition shown by the DCM leaf extracts of *Abrus precatorius* subsp. *africanus* was noteworthy as the leaves can be sustainably harvested while using the plants for medicine without threatening the survival of the plant. Anti-inflammatory activity observed for some of the investigated plant extracts in this study indicate the presence of bioactive agents that warrant further investigation. However, before biologically active compounds can be isolated from the plants that showed high inhibition of COX-2 in this study, it would be ideal to test the plant extracts for their COX-1 and LOX inhibition. Therefore, more studies (COX-1 and LOX) aimed at evaluating further the *in vitro* anti-inflammatory property of these plants is required.
Chapter 4: Genotoxicity and cytotoxicity evaluation of biologically active extracts from plants used traditionally for treating tuberculosis and related symptoms in South Africa

4.1. Introduction

Medicinal plants have been used since antiquity in the traditional treatment of various diseases and ailments (VERSCHAEVE et al., 2004). They are still used by a large proportion of the population in developing countries. Although medicinal plants have been used in therapy for many years, that does not mean that they are safe, as they may be hazardous in the long term. They are consumed with little or no proof or knowledge on their safety (CÂNDIDO-BACANI et al., 2013). Generally, plants are sessile autotrophs with the ability to adapt to challenges such as herbivores and pathogens (WINK and VAN WYK, 2008). They defend themselves against these challenges by producing a range of toxic substances that are released in response to specific environmental stimuli which in nature act as defence mechanisms against pathogenic infections, insects and herbivores. However, they can also be harmful to human beings, and thus medicinal plants should be used with caution (CUZZOLIN et al., 2006).

Toxicity occurs when a substance exerts adverse effects on an organism and its metabolism (WINK and VAN WYK, 2008). Neurotoxins affect the nervous system and are the most poisonous substances, followed by cytotoxins and metabolic poisons that affect the liver, heart, kidneys, respiratory system, muscles and reproduction (WINK and VAN WYK, 2008). Neurotoxins affect important ion channels of neuronal cells either by permanently activating or deactivating them, and cytotoxins interfere with important cellular functions (WINK and VAN WYK, 2008).

Paracelsus, “the father of toxicology”, wrote that “the dose makes the poison”, explaining that dose differentiates a poison from a remedy. In other words, an ordinarily harmless substance can be poisonous if consumed in large amounts and, conversely, substances considered toxic may be harmless in small doses (HAYES, 2008). A Greek pharmacist and physician, Galen,
demonstrated that plants do not only contain beneficial medicinal constituents, but they also have toxic substances (IFEOMA and OLUWAKANYINSOLA, 2013). Several studies have led to the validation of some traditional remedies. However, research has demonstrated that natural products are potentially toxic, carcinogenic and teratogenic in both in vitro and in vivo assays, thus they should be used cautiously (ELGORASHI et al., 2003; VERSCHAEVE et al., 2004; SANTIN et al., 2011). Concerns have been raised about the toxicity and side effects of medicinal plants in long term use. The WHO continues to encourage the use of medicinal plants in developing countries to supplement their healthcare program provided that they are proven to be non-toxic (HONG and LYU, 2011). The potential toxicity effects of some of the more popularly used herbal remedies are a cause for concern. It is thus necessary to assess the mutagenicity and cytotoxicity of traditional medicinal plants to ensure their relative safety as it would be dangerous to assume that all plant extracts are safe to use.

4.2. Mutagenicity

Mutagenesis is defined as a process by which the amount or structure of genetic material of an organism or cell is changed in a stable, heritable manner, either in nature by the use of chemicals or radiation (MORTELMANS and ZEIGER, 2000; ABDELMIGID, 2013). The genome can be damaged either spontaneously or from exposure to genotoxic agents. Mutagens are chemicals responsible for induction of mutations. These changes may include a single gene, blocks of genes, or whole chromosomes. Clastogenicity refers to effects of agents that result in structural chromosome abnormalities (chromosome rearrangement or loss); and aneugenicity refers to the effects of agents that give rise to a change (gain or loss) in chromosome number in cells (ABDELMIGID, 2013).

4.3. Negative effect of mutagenicity in human beings

Chemicals that are capable of inducing mutations even at low exposure levels can potentially damage germ lines resulting in negative effects to human health. Somatic mutations occurring in proto-oncogenes, tumor suppressor genes and/or DNA response genes, have been reported to
result in cancer and various other genetic diseases (ABDELMIGID, 2013). DNA damage accumulation in somatic cells has been reported to play a role in accelerated aging, immune dysfunction, cardiovascular, and neurodegenerative diseases. Germ cell mutations can result in spontaneous abortions, infertility or heritable damage to offspring and possibly to subsequent generations (MORTELMANS and ZEIGER, 2000). Due to the adverse effects that genetic damage can have to human health, such as the cancer causing capability of mutagens, the evaluation of substances that can alter genetic potential of human beings has become an important procedure in safety assessment.

4.4. Genotoxicity and cytotoxicity

Generally, genotoxicity is defined as “the harmful action on the genetic material of a cell affecting its integrity”. In a broader sense, similar to mutagenicity, genotoxicity is the ability of compounds to interact with DNA or cellular apparatus such as spindle apparatus and topoisomerase enzymes which control the genome (SRIVIDYA, 2012). Substances that specifically cause genetic mutations in a cell are called genotoxins or genotoxic agents, and they are potentially carcinogenic or mutagenic. Many genotoxic events do not become evident as mutations, because cells have the capacity to protect themselves from potential mutagenic effects (SRIVIDYA, 2012). However, the ability to damage the genome is an indicator of potential mutagenicity. Therefore, some techniques used for genotoxicity testing may not provide direct evidence of heritable mutation.

Cytotoxicity is defined as the negative effects that occur as a result of interferences with structures and processes that are vital for survival, proliferation and/or function of cells (RAZAK et al., 2007). These negative effects may include loss of membrane integrity, cellular metabolism, synthesis and degradation or release of cellular constituents, ion regulation as well as cell division (RAZAK et al., 2007).

For any toxicological assay on medicinal plants, the main aim is to identify negative effects and determine the limit of exposure at which such effects can occur as well as to detect toxic plant extracts in early and late stages of drug discovery and development (SRIVIDYA, 2012). There
are many bioassays that are available for determining the genotoxicity and cytotoxicity of medicinal plants (RATHNASAMY et al., 2013).

4.4.1. Genotoxicity testing methods

Genotoxicity evaluation can be defined as *in vitro* or *in vivo* tests that are done to identify potential genotoxic carcinogens and germ cell mutagens (KIM et al., 2013). When doing genotoxicity tests, whether *in vitro* or *in vivo*, it is important to generate information on gene mutation, and changes to chromosomal structures and numbers, to provide comprehensive coverage of the mutagenic potential of a chemical (JENA et al., 2002; KIM et al., 2013). The main aim of genotoxicity testing is to exclude or identify potential mutagenic hazards to humans. In view of the fact that somatic mutational events in humans are caused by the use of plants whose toxicological characteristics are unknown, different techniques have been developed to investigate genotoxicity and antigenotoxicity (VERSCHAEVE and VAN STADEN, 2008). The genotoxicity test batteries that are recommended by regulatory agencies to detect genotoxic carcinogens both *in vitro* and *in vivo* include bacterial, yeast and mammalian studies (JENA et al., 2002). The *Bacillus subtilis* Rec-assay and the reversion assay in *Salmonella typhimurium* are the two main systems used in bacteria. The *Bacillus subtilis* Rec-assay was specifically designed to assess genotoxicity of environmental mutagens, and positive results usually indicate a covalent binding to DNA or a chemical breakage of DNA (TAKIGAMI et al., 2002). On the other hand, positive results in the *Salmonella typhimurium* reversion assay often parallel the carcinogenic potential of compounds and detect frame-shift mutations as well as base-pair substitutions (MARON and AMES, 1983). Numerous genotoxicity tests in yeasts have been developed to detect gene mutations in eukaryotes. Comparable to bacteria, some of them are based on reverse mutations in genetically modified yeast strains (GENG et al., 2012). However, others monitor the activation of DNA damage-induced over-expression of reporter genes including green fluorescent protein, enhanced green fluorescent protein and luciferase. The mammalian cell-based genotoxicity tests are often designed to detect DNA damage, gene mutation or cellular DNA damage responses (GENG et al., 2012). Several available mammalian cell-based assays are available, and they include cell lines of Chinese hamster V97 and CHO cells, human lymphoblastoid TK6 and mouse lymphoma L5178Y cells. Only three genetic loci
are validated and used widely, and these are hypoxanthine-guanine phosphoribosyltransferase, thymidine kinase and the cell membrane Na+/K+ ATPase gene. However, the problem is low sensitivity in these mammalian cell-based gene mutation assays (ENG et al., 2012).

The Ames test (microsome assay) is a short-term bacterial assay that is commonly used to detect genotoxic carcinogens which cause gene mutation, such as base pair substitutions and small frame-shifts (MORTELANS and ZEIGER, 2000; KIM et al., 2013). The test is one of the two assays that are recommended by the United Kingdom Expert Advisory Committee on mutagenicity to be appropriate as an initial screen to evaluate the genotoxicity of new chemicals and drugs (JENA et al., 2002). The test uses several Salmonella typhimurium strains, such as TA97, TA98, TA100, TA102, TA104, TA1535 and TA1537 (MORTELANS and ZEIGER, 2000). Each strain is genetically different, so using several strains in a test increases the likelihood of detecting a mutagenic chemical. Bacterial strains used in the Ames test carry a faulty (mutant) gene that prevents the bacteria from synthesising histidine that is needed for growth of colonies in the standard bacterial culture medium (MORTELANS and ZEIGER, 2000). Thus, the bacterial tester strains can only survive and grow on excess histidine-containing medium. The gene’s function can be restored when new alterations occur at the site of preexisting ones and allow cells to synthesise histidine. The new mutated cells can grow and form colonies in the absence of histidine (MORTELANS and ZEIGER, 2000; ABDELMIID, 2013). Therefore, the assay uses this principle to evaluate if the extracts or drugs can revert the mutations to restore the normal colony formation in the medium.

4.4.2. Cytotoxicity testing methods

With regard to in vitro cell culture systems, when a substance interferes with the attachment of cells, it alters the morphology and the rate of cell growth, or causes them to die, it is then considered to be cytotoxic (McGAW et al., 2014). Cytotoxicity testing allows identification and prioritisation of plant extracts useful for further biological evaluation. A lot of attention has been devoted to cytotoxicity studies as a first research step in toxicity evaluation of plant extracts and active compounds isolated from plants. Minimal to no toxicity is essential for bioactive purified
compounds or plant extracts identified for pharmaceutical use, however, high toxicity intended for cancerous cells is essential.

Cytotoxicity can be measured in various ways, including evaluation of net change in population size, cell mass or metabolic activity (Fotakis and Timrell, 2006). In general, the criteria for measuring viability of cell cultures can be divided in terms of the used indicator and evaluation method. The most commonly used assays for measuring cytotoxicity or cell viability following exposure to toxic substances are lactate dehydrogenase (LDH) leakage, protein quantification, neutral red and mitochondrial reduction (MTT) assays (Mosmann, 1983; Fotakis and Timrell, 2006). The LDH leakage assay measures LDH activity in the extracellular medium. This assay has been used as a cytotoxicity indicator in HepG2 cells after exposing them to cadmium chloride, and in toxicity studies using rat renal proximal tubular cells (Fotakis and Timrell, 2006). The characteristics of the LDH leakage assay are reliability and speed.

The neutral red assay is used to provide a quantitative measure of viable cells in cultures (Fotakis and Timrell, 2006). This assay is based on the ability of living cells to take up neutral red and incorporate it within the lysosomes. Primary cells and cell lines from diverse origins are used in this assay. It is one of the most used cytotoxicity tests with many biomedical and environmental applications.

The protein assay measures cytotoxicity indirectly by measuring protein content of viable cells following the exposure of exponentially growing cultures of cells to test materials (compound or crude extract) (Fotakis and Timrell, 2006).

The MTT assay is a rapid colorimetric test that was designed to measure only living cells (Mosmann, 1983). The assay is based on the ability of mitochondrial succinate dehydrogenase enzymes of metabolically active cells to reduce [3-(4,5-dimethylthiazol-2-yl)-2-5diphenyltetrazolium bromide] or MTT to a water insoluble purple formazan (Mosmann, 1983). Many salts are used to detect viability of cells, however, MTT is the most commonly used
salt. The MTT is a water soluble tetrazolium salt that is converted to formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria (FOTAKIS and TIMBRELL, 2006). Formazan accumulates in healthy cells, and is impermeable to cell membranes. The results for this assay can be read on a multiwell spectrophotometer (ELISA reader). The MTT tetrazolium reduction assay is the first homogenous cell viability test that was developed for a 96 well microplate and is suitable for high throughput cytotoxicity screening (MOSMANN, 1983). This assay is versatile and quantitative, and is thus a “gold” standard to which new viability or cytotoxicity assays are compared. It requires incubation of a reagent (plant extract/ or compound) with a population of viable cells to convert a substrate to a colored product that can be detected with a microplate reader. The MTT substrate is prepared in physiologically balanced solution and incubated for 1 to 4 h. Only viable cells can reduce MTT, so the quantity of formazan reduced is directly proportional to the intensity of color and cell viability. As evidenced from various published articles, this assay is widely used and is popular in academic laboratories for cytotoxicity evaluation of plant extracts or compounds (McGAW et al., 2007; MUKANDIWA et al., 2012; ADEROGBA et al., 2014; MAKHAFOLOA et al., 2014).

Traditional medicinal plant use and prescription in South Africa is not regulated, and this poses a danger of misadministration, especially if plants are toxic (FENELL et al., 2004). In Chapter 2, several plant extracts were reported to exhibit good antibacterial activities with MIC values ranging from 0.098-0.78 mg/ml against any of the two Mycobacterium species (Mycobacterium aurum A+ and Mycobacterium tuberculosis) or bacterial strains associated with respiratory infections (Staphylococcus aureus and Klebsiella pneumoniae) tested. Based on those findings, further genotoxicity and cytotoxicity investigations were carried out on the antimicrobial extracts with MIC values ranging from 0.098-0.78 mg/ml. These tests are necessary to determine whether the antimicrobial as well as other biological activities of medicinal plants are due to their toxicity or not. Plants showing clear toxicity should be considered as potentially unsafe and must be tested further before their continued use can be recommended. Those with clear non-toxicity potential together with antimicrobial activity should be considered noteworthy for therapeutic use and should also be investigated further for their pharmacological properties (VERSCHAEVE and VAN STADEN, 2008).
4.5. Materials and methods

4.5.1. Preparation of plant extracts for genotoxicity testing

Plant materials were extracted as described in Section 2.5.2. To prepare the aliquots, biologically active extracts were dissolved in 10% DMSO to make a concentration of 5000 μg/ml and filter sterilised through 0.22 μm filters. After filter sterilisation, the 5000 μg/ml concentration of the sample extracts was diluted with sterile 10% DMSO to obtain lower concentrations of 500 and 50 μg/ml.

4.5.2. In vitro genotoxicity evaluation of biologically active extracts using the Ames test

The genotoxicity evaluation of biologically active plant extracts was done in histidine deficient growth medium using the *Salmonella* microsome assay. The *Salmonella* microsome assay was used to test mutagenicity based on the plate-incorporation procedure. The Ames test was performed by incorporating test extracts with *Salmonella typhimurium* tester strains TA98 and TA100 without metabolic activation. The procedure was done according to MARON and AMES (1983) modified by MORTEL MANS and ZEIGER (2000). Bacterial stocks (100 μl) were incubated in 20 ml of Oxoid No.2 nutrient broth at 37 °C on a rotary shaker for 16 h. The cultured bacteria (100 μl) were added to 100 μl of plant extract with 500 μl of phosphate buffer and 2 ml of top agar containing biotin-histidine (0.5mM). The mixture was mixed by vortexing, then gently poured over the surface of a minimal agar plate and incubated at 37 °C for 48 h. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 μg/ml whereas sterile distilled water was the negative control. All the samples were tested in triplicate. The number of bacterial colonies was counted using a colony counter after 48 h of incubation and the results were expressed as the mean (±standard error) number of the revertant colonies per plate. The absence of toxicity was examined by observing background bacterial growth, which should normally be present.
4.5.3. *In vitro* cytotoxicity testing of biologically active extracts using the MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to MOSMANN (1983) was used to evaluate viability of cells after their exposure to the test substances. The biologically active extracts in Chapter 2 were tested for cytotoxicity against the Vero (African green monkey kidney) cell line obtained from the Department of Veterinary Tropical Diseases, University of Pretoria.

The Vero cells were cultured in sterile Minimal Essential Medium (MEM, Sigma) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (FCS, Sigma) in a 75 cm² flask incubated at 37 °C in 5% CO₂. After one week of incubation, the medium was decanted, and the cells adhering to the bottom of the flask were washed twice with 20 ml of phosphate buffered saline (PBS, Sigma). Following the washing step, trypsin (3 ml) was added to the cells which were then incubated at 37 °C for up to 20 min to allow detachment of cells from the flask. When the cells had detached from the flask, MEM containing FCS was added to stop the enzymatic action of the trypsin. The cells were poured into centrifuge tubes and centrifuged for 2 min at 200 x g. A Neubauer haemocytometer was used to count the number of cells per ml. Trypan blue was added to determine the number of viable cells as viable cells extrude the trypan blue and appear clear while dead cells are blue. The cells were resuspended to a concentration of 1 x 10⁵ cells/ml and 100 µl were added to each well of columns 2 to 11 of 96-well microtitre plates at a final concentration of 10 000 cells per well. Minimal Essential Medium (200 µl) was added to wells of columns 1 and 12 to maintain humidity. The microplates were incubated overnight at 37 °C in a 5% CO₂ incubator until the cells were in the exponential phase of growth, to allow cell attachment to the bottom of the microplates. The attached cells in 96 well microplates were used for the cytotoxicity assay.

The bioactive plant extracts were resuspended in ethanol/DMSO to a concentration of 100 mg/ml and filter sterilised before being diluted in fresh MEM to the required concentrations. The concentrations tested ranged from 0.0075 to 1 mg/ml. The plant extract dilutions (100 µl) were pipetted into wells of the microplates in quadruplicate. Doxorubicin hydrochloride (Sigma) was...
used as a positive control and solvent controls were also included. The microplates were incubated at 37 °C in a 5% CO₂ incubator for 48 h. Following incubation with test extracts, the cells were examined using an inverted microscope to detect cytopathic effects (CPE). Minimal Essential Medium containing plant extracts was removed and each well was rinsed with PBS immediately, and fresh MEM (200 µl) without test substance was added to the wells. A concentration of 5 mg/ml of MTT in PBS was prepared, and 30 µl of the solution were then added to each well, and the plates were incubated for a further 4 h at 37 °C in 5% CO₂ incubator. The medium was removed from the wells after 4 h incubation and 50 µl of DMSO were added to each well to dissolve the formazan crystals. The plates were gently shaken until the solution was dissolved prior to reading. The amount of MTT reduction was evaluated by measuring the absorbance at a wavelength of 570 nm (reference wavelength of 630 nm) in a microplate reader. The wells without cells which contained only the medium were used to blank the plate reader. The results were interpreted as a percentage of the control wells and the LC₅₀ (lethal concentration 50) values were calculated. The selectivity index values for each extract were calculated using the following formula:

\[
\text{Selectivity index} = \frac{\text{LC₅₀ (mg/ml)}}{\text{MIC value (mg/ml)}}
\]

Where LC₅₀ stands for the concentration of a given agent (plant extract) which is lethal to 50% of the cells, and MIC stands for minimum inhibitory concentration.

4.6. Results and discussion

4.6.1. Genotoxicity

Considering the antimicrobial results (ranging from 0.098-0.78 mg/ml) obtained in Chapter 2, the activity may be due to the presence of biologically active or toxic compounds. Therefore, genotoxicity testing was done. The results of the genotoxicity assay on plant extracts with MIC values less than 1.0 mg/ml are presented in Table 4.1. The Ames test without metabolic activation is designed only for direct mutagen detection. To designate a substance as a mutagen, a positive response in any single bacterial strain either with or without metabolic activation is
sufficient (ZEIGER, 2001). TA98 and TA100 bacterial strains are often used as they detect the great majority of mutagens (VERSCHAEVE and VAN STADEN, 2008). Positive results from Salmonella typhimurium strain TA98 detects frame-shift mutations based on spontaneous reversion of the Salmonella typhimurium from His− to His+ caused by crude plant extracts. Positive results caused by TA100 indicate base-pair substitution. The evaluated extracts must exhibit a dose-dependent increase in the number of revertants in order to be considered as genotoxic. Furthermore, the number of revertant colonies of the extracts must be equal to or greater than two times that of the negative control (MARON and AMES, 1983). However, in our study none of the tested extracts demonstrated a dose-dependent increase nor revertant colonies that were equal to or greater in number than twice those of the negative control. Therefore, the tested plant extracts lacked direct genotoxic compounds. In all cases, the values of the negative as well as the positive control were within normal limits and in accordance with literature (ELGORASHI et al., 2003).

The lack of genotoxicity observed with the Ficus sur root EtOH extract against Salmonella typhimurium TA98 in our study is in accordance with that reported by ELDEEN et al. (2005b). The leaf methanol extract of Abrus precatorius subp. africanus was reported to be non-mutagenic against Salmonella TA677 in a study by MARTINEZ et al. (2012). For more in depth investigations, subsequent tests should be conducted with other strains such as TA97, TA102, TA104, TA1535 and TA1537 (MORTELmans and ZEIGER, 2000).
Table 4.1: Number of revertant colonies of *Salmonella typhimurium* strains TA98 and TA100 induced by extracts of some plants used as remedies for the treatment of tuberculosis and related symptoms in South Africa

<table>
<thead>
<tr>
<th>Plant species (part used)</th>
<th>Bioactive extract</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TA98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5000</td>
</tr>
<tr>
<td><strong>Extract concentration in µg/ml</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Abrus precatorius</em> subp. <em>africanus</em> (L)</td>
<td>Dichloromethane</td>
<td>24.33±0.33</td>
</tr>
<tr>
<td><em>Abrus precatorius</em> subp. <em>africanus</em> (L)</td>
<td>Ethanol</td>
<td>26.33±0.88</td>
</tr>
<tr>
<td><em>Abrus precatorius</em> subp. <em>africanus</em> (S)</td>
<td>Ethanol</td>
<td>28.33±3.33</td>
</tr>
<tr>
<td><em>Asparagus africanus</em> (L)</td>
<td>Ethanol</td>
<td>26.67±0.89</td>
</tr>
<tr>
<td><em>Asparagus falcatus</em> (L)</td>
<td>Ethanol</td>
<td>20.33±2.60</td>
</tr>
<tr>
<td><em>Ficus sur</em> (R)</td>
<td>Ethanol</td>
<td>22.00±1.00</td>
</tr>
<tr>
<td><em>Indigofera arrecta</em> (L)</td>
<td>Ethanol</td>
<td>21.00±2.31</td>
</tr>
<tr>
<td><em>Indigofera arrecta</em> (L)</td>
<td>Water</td>
<td>21.67±1.20</td>
</tr>
<tr>
<td><em>Leonotis intermedia</em> (L)</td>
<td>Ethanol</td>
<td>26.00±1.15</td>
</tr>
<tr>
<td>Plant species (part used)</td>
<td>Bioactive extract</td>
<td>Bacterial strains</td>
</tr>
<tr>
<td>--------------------------</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>TA98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extract concentration in µg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5000</td>
</tr>
<tr>
<td><em>Pentanisia prunelloides</em> (L)</td>
<td>Ethanol</td>
<td>20.33±1.76</td>
</tr>
<tr>
<td><em>Pentanisia prunelloides</em> (L)</td>
<td>Water</td>
<td>20.15±2.52</td>
</tr>
<tr>
<td><em>Pentanisia prunelloides</em> (R)</td>
<td>Ethanol</td>
<td>26.67±1.26</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (L)</td>
<td>Ethanol</td>
<td>28.00±0.57</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (L)</td>
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</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (R)</td>
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<td>23.00±1.15</td>
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<tr>
<td><em>Terminalia phanerophlebia</em> (R)</td>
<td>Water</td>
<td>22.15±1.52</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (T)</td>
<td>Ethanol</td>
<td>28.67±0.89</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (T)</td>
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<td>Water</td>
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<tr>
<td>4NQO</td>
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<td>133.00±1.21</td>
</tr>
</tbody>
</table>

4.6.2. Cytotoxicity

Cytotoxicity results of 18 biologically active crude extracts (in Chapter 2) from eight plant species are presented in Table 4.2. The selectivity index is the ratio of cytotoxicity to biological activity. When a plant extract has a selectivity index value greater than one, it is more active against the target bacterial strain and less toxic to the mammalian cells used in the cytotoxicity assay. When its selectivity index value is less than one, it is more toxic and less active. In our study the selectivity index values for the tested extracts ranged between 0.04 to 16.43. The leaf water extract of *Terminalia phanerophlebia* showed the highest selectivity index value of 16.43, which was noteworthy as the extracts from this plant part showed good antimicrobial activity. For *Terminalia phanerophlebia*, it was disturbing that the EtOH and water extracts of each plant part differed to a large extent in their toxicity. However, the leaf EtOH extracts of *Terminalia phanerophlebia* were reported to have selective toxicity when tested against human kidney epithelial cells in a study done by SIBANDZE (2009). The results indicate that even if both extractants are highly polar, it does not necessarily mean that the EtOH extracts would be as safe as water extracts that are used traditionally.

For *Pentanisia prunelloides* leaf and root extracts, the selectivity index values were greater than one for all the bioactive extracts tested, ranging from 1.05 to 3.38. This means that the extracts showed more activity against the bacterial strains and less toxicity to the mammalian cells. The crude extract of *Pentanisia prunelloides* roots was reported to have more potency and less toxicity when studied using brine shrimp lethality tests (MPOFU, 2013).

The EtOH extracts of *Abrus precatorius* subsp. *africanus* (leaves) and *Asparagus africanus* (leaves) demonstrated lower toxicity and more antimicrobial activity with a selectivity index values ranging from 1.05 to 4.97. The leaves of *Abrus precatorius* subsp. *africanus* were reported to have caused spastic paralysis and death of chickens within 24 h; therefore this plant should be administered with caution (MARTINEZ et al., 2012).

The seed EtOH extracts of *Abrus precatorius* subsp. *africanus* demonstrated greatest toxicity out of all the extracts tested with a selectivity index value of 0.04. This is not surprising as
MARTINEZ et al. (2012) reported poisonous effects of extracts from the seeds of *Abrus precatorius* subsp. *africanus* in an *in vivo* study. A total of 20 crushed seeds of *Abrus precatorius* subsp. *africanus* mixed with water and taken orally by adult males were reported to have caused vomiting of blood, pain in the eyes and burning of ears (ROSS, 2003). Seeds of *Abrus precatorius* subsp. *africanus* were reported to be poisonous due to the presence of abrin, a toxic protein, and an alkaloid hypaphorine (SINGH et al., 1998). Bruquinone G previously isolated from the aerial parts of *Abrus precatorius* subsp. *africanus* were reported to have demonstrated mild toxicity (LIMMATVAPIRAT et al., 2004). Therefore caution must be taken when using this plant species.

This study demonstrated a lack of safety for *Abrus precatorius* subsp. *africanus* (seed EtOH and leaf DCM extracts), *Asparagus falcatus*, and *Indigofera arrecta* (leaf EtOH extract) with selectivity index values lower than one, ranging from 0.04 to 0.95. *Leonotis intermedia* (leaves) and *Ficus sur* (roots) EtOH extracts showed some toxicity with selectivity index values of 0.33 and 0.96 respectively. Therefore caution should be exercised when using these plants for medicinal purposes. However, in an *in vivo* study by NYARKO et al. (1999) the leaf extracts of *Indigofera arrecta* were reported to be devoid of acute and subchronic toxicity. *Ficus sur* was also reported to be devoid of cytotoxicity against brine shrimp (MOSHI et al., 2007). Therefore, more toxicity studies are required to evaluate the safety of these plants.

Some of the plant extracts tested demonstrated selectivity index values lower than 1. However, it should be noted that *in vitro* toxicity does not always equate to *in vivo* toxicity because of the difference in microenvironment and pharmacodynamic interactions in live animals and tissue culture (FRESHNEY, 2000). Metabolism is one of the critical factors in toxicology as some substances demonstrating non-toxicity initially may produce toxic metabolites after being exposed to liver enzymes, while other substances that are toxic *in vitro* may become detoxified (McGAW et al., 2014). Generally, in *in vitro* toxicity evaluation, toxic responses are determined by measuring the changes in cell survival or metabolism, therefore models that are more closely related to tissue or systemic toxicity should be considered. It should be taken into consideration that *in vivo* toxicity might be due to a tissue response, probably caused by an inflammatory reaction. The low cytotoxicity observed against Vero cells for some of the biologically active
extracts tested in this study were noteworthy, and more tests are required to verify the lack of toxicity demonstrated by these extracts.
Table 4.2: Average LC$_{50}$, and selectivity index values of bioactive plant extracts

<table>
<thead>
<tr>
<th>Plant species (part used)</th>
<th>Bioactive extracts</th>
<th>Average LC$_{50}$ and Standard Deviation</th>
<th>Minimum inhibitory concentration values (mg/ml)</th>
<th>Selectivity index values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
<td>K. p</td>
<td>S. a</td>
</tr>
<tr>
<td><em>Abrus precatorius</em> subsp. <em>africanus</em> (L)</td>
<td>EtOH</td>
<td>0.97 ± 0.04</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Abrus precatorius</em> subsp. <em>africanus</em> (L)</td>
<td>DCM</td>
<td>0.16 ± 0.01</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Abrus precatorius</em> subsp. <em>africanus</em> (S)</td>
<td>EtOH</td>
<td>0.03 ± 0.00</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Asparagus africanus</em> (L)</td>
<td>EtOH</td>
<td>0.46 ± 0.00</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Asparagus falcatus</em> (L)</td>
<td>EtOH</td>
<td>0.28 ± 0.01</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Ficus sur</em> (R)</td>
<td>EtOH</td>
<td>0.75 ± 0.03</td>
<td>0.78</td>
<td>0.195</td>
</tr>
<tr>
<td><em>Indigofera arrecta</em> (L)</td>
<td>EtOH</td>
<td>0.37 ± 0.03</td>
<td>0.78</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Indigofera arrecta</em> (L)</td>
<td>Water</td>
<td>&gt;1.00 ± 0.00</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Leonotis intermedia</em> (L)</td>
<td>EtOH</td>
<td>0.26 ± 0.00</td>
<td>na</td>
<td>0.78</td>
</tr>
<tr>
<td>Plant species (part used)</td>
<td>Bioactive extracts (mg/ml)</td>
<td>Average LC&lt;sub&gt;50&lt;/sub&gt; and Standard Deviation</td>
<td>Minimum inhibitory concentration values (mg/ml)</td>
<td>Selectivity index values</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
<td>K. &lt;i&gt;p&lt;/i&gt;</td>
<td>S. &lt;i&gt;a&lt;/i&gt;</td>
</tr>
<tr>
<td><em>Pentanisia prunelloides</em> (R)</td>
<td>EtOH</td>
<td>0.82 ± 0.18</td>
<td>0.39</td>
<td>0.78</td>
</tr>
<tr>
<td><em>Pentanisia prunelloides</em> (L)</td>
<td>Water</td>
<td>&gt;1 ± 0.00</td>
<td>na</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Pentanisia prunelloides</em> (L)</td>
<td>EtOH</td>
<td>0.66 ± 0.06</td>
<td>na</td>
<td>0.195</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (L)</td>
<td>EtOH</td>
<td>0.08 ± 0.01</td>
<td>0.195</td>
<td>0.195</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (L)</td>
<td>Water</td>
<td>1.61 ± 0.52</td>
<td>0.39</td>
<td>0.098</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (T)</td>
<td>EtOH</td>
<td>0.25 ± 0.09</td>
<td>0.195</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (T)</td>
<td>Water</td>
<td>&gt;1 ± 0.00</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (R)</td>
<td>EtOH</td>
<td>0.17 ± 0.01</td>
<td>na</td>
<td>0.195</td>
</tr>
<tr>
<td><em>Terminalia phanerophlebia</em> (R)</td>
<td>Water</td>
<td>&gt;1 ± 0.00</td>
<td>na</td>
<td>0.39</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td>6.78 ± 0.39</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

L: leaves, R: roots, B: bark, S: seeds, T: twigs, DCM: dichloromethane, PE: petroleum ether, EtOH: ethanol, K. <i>p</i>: **Klebsiella pneumoniae**, S. <i>a</i>, *Staphylococcus aureus*, M. <i>a</i> A+, *Mycobacterium aurum* A+, M. <i>tb</i> H37Ra, *Mycobacterium tuberculosis*, na, extract not active (MIC value greater than 1 mg/ml) against a particular strain and selectivity index value (-) not determined. Doxorubicin hydrochloride in µg/ml (positive control), Minimum inhibitory concentration values of extracts (mg/ml) from Chapter 2, LC<sub>50</sub> = Lowest concentration of extract which is lethal to 50% of the cells.
The low toxicity (genotoxicity and cytotoxicity) and promising antimicrobial results demonstrated by *Pentanisia prunelloides* in this study endorse the traditional therapeutic use of this plant species in South Africa. It is evident that more tests are necessary for the investigation of the toxic effects of extracts from the plants tested in this study and their interactions with cell metabolism, because no single test can detect every toxin.

### 4.7. Conclusions

Non-genotoxic activity demonstrated by the evaluated plant extracts does not confirm that they are completely safe for consumption as their metabolites could be genotoxic. It confirms that the substance is not genotoxic to a particular bacterial strain against which it is tested, and for the genetic end point tested. Non-genotoxicity in the extracts of interest in this study is a positive step forward in determining their safe use in the treatment of tuberculosis and related symptoms. Lack of cytotoxic effects to Vero cells at the highest concentration tested of 1 mg/ml of some of the bioactive extracts was noteworthy. *Abrus precatorius* subsp. *africanus*, *Asparagus falcatus*, *Leonotis intermedia*, *Ficus sur*, *Indigofera arrecta* as well as *Terminalia phanerophlebia* should be administered with caution as some of their selectivity index values were lower than one in this study. However, more studies are required to verify lack of genotoxicity and more in depth cytotoxicity tests against other cell lines are needed prior to venturing into investigation of *in vivo* toxicity.
Chapter 5: Isolation and characterisation of antimicrobial compounds from *Terminalia phanerophlebia* Engl. & Diels leaf extracts

5.1. Introduction

An emergency “situation” was declared by the WHO in 2005, for urgent action to stop the worsening epidemic of tuberculosis in Africa (FYHRQUIST et al., 2014). According to the WHO estimations, between 1990 and 2005, in African countries the average incidence of tuberculosis had more than doubled (WHO, 2010). Tuberculosis epidemics are aggravated by the emergence of drug resistance and HIV coinfection. To counteract this problem, there is a need to find new and effective tuberculosis treatments that will fight challenges such as toxicity, drug-resistance, as well as coinfection and will reduce the duration of treatment to cure the disease. GUPTA et al. (2014) reported that, diverse compounds with potent antimicrobial activity were isolated from higher plants. Therefore, drugs that might act as an effective treatment of tuberculosis could be found in medicinal plants.

The family Combretaceae is placed in the Myrtales and comprises of approximately 20 genera and 600 species of trees, shrubs and lianas (ELOFF et al., 2008; DAWE et al., 2013). Plant species belonging to the Combretaceae are widespread in tropical and subtropical regions of the world specifically in forests, grassland and mangroves, mostly in Africa and India (TILNEY, 2002; ELOFF et al., 2008; DAWE et al., 2013). Genera of the Combretaceae family includes: Anogeissus, Buchenavia, Bucida, Calopyxis, Calycoperis, Combretum, Conocarpus, Dansiea, Guiera, Languncularia, Lumnitzera, Macropteranthes, Melostemon, Pteleopsis, Quisqualis, Stace, Strephonema, Terminalia and Thiola. In southern Africa, the Combretaceae is represented by seven genera and these include Combretum, Lumnitzera, Meiostemon, Stace, Pteleopsis, Quisqualis and Terminalia (TILNEY, 2002). *Combretum* and *Terminalia* are the largest two genera in the Combretaceae.

Several species in the Combretaceae family are used in many regions of the world to cure various diseases and ailments. Some of the uses include: treatment of tuberculosis, pneumonia, coughs, colds, fever, cancer, abdominal disorders, acute enteritis, dysentery,
diarrhoea, hook worm, constipation, gastric ulcers, bilharzia, venereal disease, eye diseases, heart diseases, urinary system cleansing, syphilis, beriberi, toothache, jaundice, leprosy, malaria, gingivitis and temporary insanity (TILNEY, 2002).

The genus *Terminalia* consists of approximately hundred species that are distributed in the tropical regions of the world (KUETE et al., 2010b). Approximately 30 of the *Terminalia* species are distributed in Africa and 11 are from the southern region of the continent. The plants range from shrubs to big trees occurring in dry woodlands and rainforests (FYHRIQUIST et al., 2014). The 11 southern African species include: *Terminalia pruniodes* M.A. Lawson, *Terminalia randii* Baker f., *Terminalia stuhlmannii* Engl., *Terminalia brachymstemma* Welw. ex Hiern, *Terminalia sericea* Burch. ex DC., *Terminalia trichopoda* Diels, *Terminalia gazensis* Baker f., *Terminalia phanerophlebia* Engl. & Diels, *Terminalia molli* Exell, *Terminalia sambesiaca* Engl. & Diels and *Terminalia stenostachya* Engl. & Diels (ELOFF et al., 2008). Species of *Terminalia* are widely used as medicinal plants both in Africa as well as in Asia and several are reported to exhibit biological activities (MASOKO and ELOFF, 2005). Several studies on the pharmacological activities of *Terminalia* have proven the plant species from this genus to contain antimicrobial, antiviral, anti-inflammatory, antioxidant and anthelmintic activities (MASOKO and ELOFF, 2005).

### 5.2. Traditional uses and pharmacological studies of *Terminalia phanerophlebia*

Different parts (roots, barks and leaves) of *Terminalia phanerophlebia* are used in TM for treatment of various diseases such as: pneumonia, bilharzia, hypertension, cancer, diabetes, stomach problems, schistosomiasis, gonorrhoea, syphilis, gynaecological conditions, inflammation, epilepsy, sexual transmitted diseases, wounds and skin diseases (NEUWINGER, 1996; VAN WYK et al., 2000, SCHMELZER and GURIB-FAKIM, 2013). In South Africa, different cultures use roots of *Terminalia phanerophlebia* to treat witchcraft associated diseases that are believed to culminate in coughing leading to tuberculosis and rheumatism (MABOGO, 1990). Leaf decoctions are used in the traditional treatment of coughs, stomach complaints and opthalmia. The powdered leaves of *Terminalia phanerophlebia* are applied as a dressing to wounds. The widespread use of *Terminalia phanerophlebia* in indigenous medicine for the traditional treatment of different ailments
means that it represents a rich and readily available source of molecules with various kinds of biological activities, which have potential for use in pharmaceutical products. The crude extracts of the leaves, twigs and roots of this plant have demonstrated good antimicrobial activity against bacterial strains known to cause tuberculosis and opportunistic infections of the respiratory tract. Three compounds were reported from the stem bark of *Terminalia phanerophlebia* (NAIR et al., 2012). The compounds were identified as β-sitosterol, β-sitostenone and stigmast-4-ene-3,6-dione. The study demonstrated β-sitosterol as an anti-inflammatory agent.

Many reports have mentioned that *Terminalia* contains antimycobacterial property, however only a few species have been investigated for their antimycobacterial constituents (McGAW et al. 2008a; FYHRQUIST et al., 2014). In a study by GREEN et al. (2010), *Terminalia sericea* stem bark acetone extract proved to have antimycobacterial activity as it inhibited the growth of both the attenuated and a clinical strain of *Mycobacterium tuberculosis*. The leaf crude extracts of *Terminalia glaucescens* Planch was reported to inhibit the growth of a revertant *Mycobacterium tuberculosis* strain (NVAU et al., 2011). It also showed inhibitory activity against other *Mycobacterium* species such as *Mycobacterium ulcerans*, *Mycobacterium madagascariense* and *Mycobacterium indicus*. Promising antimycobacterial effects were demonstrated by root extracts of *Terminalia avicennoides* Guill. & Perr. against two *Mycobacterium* species: *Mycobacterium tuberculosis* and *Mycobacterium bovis* (FYHRQUIST et al., 2014).

According to SIBANDZE et al. (2009), species of *Terminalia* have been reported to contain several secondary metabolites with antibacterial property. Such compounds include arjunolone from *Terminalia arjuna* (Roxb. Ex DC.) Wight & Arn., punicalagin from *Terminalia oblongata* (Ruiz & Pav.) Steud., imberbic acid dirhamnosoid from *Terminalia stuhlmanii* Engl., as well as diphenoyl-gallagylglucose and ellagitannin derivatives from *Terminalia oblongata*, *Terminalia clamensae* De Wild., *Terminalia catappa* L., and *Terminalia cheluba* Retz (ELOFF et al., 2008). Resveratrol-3-β-rutinoside, anolignan B, termilignan B and arjunic acid with antibacterial effects were isolated from *Terminalia sericea* (ELDEEN et al., 2005a; 2008). Ellagitannins and pentacyclic triterpenoids have been reported to be isolated from *Terminalia macroptera* (FYHRQUIST et al., 2014).
Antimycobacterial compounds have been isolated from species of *Terminalia*, thus proving that some of the species of this genus have antimycobacterial effects. Compounds such as 3,4'-di-O-methylellagic acid 3'-O-β-D-xylopyranoside and 4'-O-galloy-3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside isolated from stem bark extract of *Terminalia superba* Engl. & Diels exhibited good antimycobacterial activity against four strains of mycobacteria (KUETE et al. (2010b)). Pentacyclic triterpenoid and fridelin isolated from the leaf extracts of *Terminalia avicennoides* showed promising inhibitory activity against *Mycobacterium bovis* (FYHRQUIST et al., 2014). Acetyl rhamnosides of 1,3-hydroxylated pentacyclic triterpenoids from *Terminalia stuhlmanii* showed good inhibition of *Mycobacterium fortuitum* (MANN et al., 2011). More studies on the isolation of compounds with antimycobacterial compounds from *Terminalia* species are needed as some of the plants from this genus have proven to have antimycobacterial constituents.

In the current studies, on *in vitro* antimicrobial and antimycobacterial, anti-inflammatory as well as genotoxicity evaluation of extracts from plants used traditionally in South Africa to treat tuberculosis and related symptoms, several plant species reported to have exhibited good antibacterial activity against *Mycobacterium* species and other bacterial strains associated with respiratory infections. *Terminalia phanerophlebia* was one of them (MADIKIZE et al., 2013b; 2014). *Terminalia phanerophlebia* leaf extracts showed considerable antimicrobial activities against bacterial strains tested and exhibited non-genotoxicity against *Salmonella typhimurium* strains. Thus, the aims of this study were to isolate and identify active antimicrobial compounds from *Terminalia phanerophlebia*.

5.3. Materials and methods

5.3.1. Bioassay guided fractionation of 80% methanol extracts of *Terminalia phanerophlebia* leaves

In isolating bioactive compounds, different methods of separation are commonly used to obtain pure compounds, such methods include, thin layer chromatography (TLC), column chromatography using silica gel or Sephadex, flash chromatography, and high performance liquid chromatography (HPLC) (SASIDHARAN et al., 2011). Thin layer chromatography is a procedure that reveals the number of components in a mixture, after viewing the plate with the sample under the ultraviolet light (254 or 366 nm) or can be used as an analytic
fingerprint of a plant extract (AZMIR et al., 2013). The different isolation techniques have a common objective: to isolate compounds (AZMIR et al., 2013). When pure compounds are obtained after isolation, they are then used for structure and biological activity determination.

Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. As a result, their separation remains a big challenge for the process of identification and characterisation of bioactive compounds. The unmatched availability of chemical diversity of plant extracts either as pure compounds or as standardised extracts provides unlimited opportunities for new drug discoveries. Bioassay-guided fractionation is a vital process in the isolation and identification of compounds with biological activity (GAUTAM et al., 2007).

5.3.2. General

All TLC analyses were performed at room temperature using pre-coated plates (MERCK, silica gel 60 F254 0.2 thickness). Detection of spots was done by viewing under ultraviolet light (254 and 366 nm). Open column chromatography was carried out using silica gel (230–400 mesh) and Sephadex LH-20. Nuclear magnetic resonance (NMR) data such as $^1$H, $^{13}$C, DEPT, COSY, NOESY, HSQC and HMBC spectra were obtained using a Bruker AV400 spectrometer or Brucker 500 MHz spectrometer. Chemical shifts are expressed in parts per million (ppm).

5.3.3. Plant material-collection and authentication

The leaves of *Terminalia phanerophlebia* were collected in February, 2013 from the UKZN botanical garden, Pietermaritzburg, South Africa. The plant was identified by Alison Young (UKZN, Horticulturist) and the voucher specimen (BALUNGI 37) was deposited in UKZN Herbarium (NU) (Pietermaritzburg, South Africa) for botanical authentication. The collected leaves were separated from stalks and oven dried at 50°C. When completely dried, the leaves were ground into powder and kept in airtight containers until use.

5.3.4. Sample extraction
The powdered plant material (1 kg) was extracted with 8 l of 80% methanol (MeOH) at room temperature for 24 h with occasional shaking and filtered through Whatman No.1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator at 40 °C. This afforded a crude extract (166.60 g) from the leaves of *Terminalia phanerophlebia*.

5.3.5. Solvent partitioning of the crude extracts

The crude extract was suspended in 500 ml water: MeOH (9:1) mixture, and then partitioned sequentially with hexane (3 × 800 ml), dichloromethane (DCM) (3 × 800 ml), ethyl acetate (EtOAc) (3×800 ml) and butanol (500 ml). The solvent fractions (hexane, DCM, EtOAc and butanol) were concentrated to dryness using a rotary evaporator and afforded four fractions. The mass of each fraction was 1.82 g (hexane), 2.59 g (DCM), 16.45 g (EtOAc) and 23.62 g (butanol) respectively. All the extracts were subsequently assayed for antimicrobial activity.

5.3.6. Isolation of compounds from *Terminalia phanerophlebia* ethyl acetate fraction

The most active fraction (EtOAc fraction, 16.45 g) was fractionated on a silica gel (230-400 mesh) column. The column was filled with silica gel by pouring it directly on top of a small cotton wool stopper that was first inserted in the column. The column with the silica gel was then washed with 100% hexane twice, 100% DCM and then finally DCM: MeOH (9:1) before adding the extract. The EtOAc extract was dissolved in a DCM: MeOH (9:1) solvent mixture. The dissolved EtOAc extract was added to silica gel dropwise. The resultant silica gel was subsequently stirred using a mortar and pestle to evaporate the solvent until the extract was completely adsorbed on silica gel. Then silica gel adsorbed extract was added into the column using a funnel and a spatula. A cotton wool stopper was soaked in DCM: MeOH (9:1) solution and then placed on top of the sample in the column, to avoid disturbing the sample surface in the column when introducing different solvents. Elution of the sample was started with 100% DCM followed by an increasing gradient of EtOAc up to 100%. This was then in turn followed by an increasing gradient of MeOH in EtOAc up to 20%. Thirteen elusion systems were slowly added in the order as shown in Table 5.1.
A total of 171 test tubes of 20 ml each were collected and analysed on TLC plates using DCM/MeOH (9:1 and 4:1) as solvent systems. These analyses yielded 10 fractions (F1-F10). An antimicrobial assay, on the fractions collected, was performed following the same procedure described in Section 2.7 to determine the MIC values of the collected fractions. Fraction 1 and Fraction 5 showed good activity against all tested bacterial strains. Fraction 1 (37.4 mg) from test tubes 61 to 63 was purified by fractional crystallisation; 100% DCM was added to the sample and left to stand for an hour. The impurities dissolved into the DCM and the sample settled. The solvent containing impurities was carefully decanted and discarded. The same procedure was repeated three times and the sample was allowed to dry. The dried sample yielded compound 1 (27.10 mg) after analysis on TLC plates using DCM/MeOH (9:1). Fraction 5 (71.00 mg) from test tubes 96 to 105 was subjected to column chromatograph on Sephadex LH 20 using EtOAc/MeOH (9:1) as an eluent followed by an increasing gradient of MeOH from 10% up to 20%. The TLC plate analysis of the test tube fractions using DCM: MeOH (5:1) solvent mixtures yielded compound 2 (3.50 mg). The isolation scheme for compound 1 and 2 is presented in Figure 5.1.
Dried plant material (1 kg) 

Extracted with 80% methanol, and concentrated

80% methanol extracts (166.6 g)

(Suspended in water: methanol; 9:1 v/v)

Liquid-liquid partitioning

Hexane

Dichloromethane

Ethyl acetate (16.45 g) Butanol

Fractions on silica gel (DCM/EtOAc/MeOH)

Fractions

1 (37.40 mg) 2 3 4 5 (71.00 mg) 6 7 8 9 10

1 Fractional crystallisation Compound 1 (27.10 mg)

2 Purification on Sephadex LH 20 (EtOAc/MeOH) Compound 2 (3.5 mg)

Figure 5.1: Fractionation scheme of the crude extracts from the leaves of *Terminalia phanerophlebia*

5.3.7. Structure elucidation of isolated compounds

Structure elucidation of the isolated compounds was carried out using NMR spectroscopic techniques: $^1$H (500 MHz) and $^{13}$C NMR (125 MHz), and DEPT together with 2D experiments (GCOSY, GHSCQ and GHMBC). The compounds were identified as:

Methyl gallate (methyl-3,4,5-trihydroxybenzoate) (Figure 5.14), $^1$H NMR (MeOD) δ: 3.81 (3H, s, –OCH3), 7.05 (2H, s, H-2,H-6). $^{13}$C NMR (125 MHz, MeOD) δ: 121.6 (C-1), 110.2 (C–H,C-2), 146.6 (C-3), 139.9 (C-4), 146.6 (C-5), 110.2 (C–H, C-6), 169.1 (C=O), 52.3 (–
OCH3). The $^1$H NMR, $^{13}$C NMR, DEPT, GCOSY, GHSQC and GHMBC spectrum of methyl-3,4,5-trihydroxybenzoate are presented in Figures 5.2 to 5.6. The spectra data are in good agreement with that of methyl-3,4,5-trihydroxybenzoate in the literature (MADIKIZELA et al., 2013a).

Compound 2: It was determined to be 1,6-di-O-coumaroyl glucopyranoside (Figure 5.14) from its spectroscopic data, NMR ($^1$H and $^{13}$C), Table 5.2.

5.3.8. Antimicrobial assay procedure

The MIC values of the crude extracts, four solvent fractions, column fractions obtained from the EtOAc sample, and isolated compounds from Terminalia phanerophlebia leaves were determined using the broth microdilution method in 96 well microtitre plates against Staphylococcus aureus and Klebsiella pneumoniae as described by ELOFF (1998a). The REMA and broth microdilution method according to JADAUN et al. (2007) modified by GREEN et al. (2010) were used to determine the antimycobacterial activity of the extracts against Mycobacterium tuberculosis H37Ra and Mycobacterium aurum A+. All the procedures were followed as described in Section 2.4 and 2.5.

5.4. Results and discussion

5.4.1. Characterisation of compounds 1 and 2

Bioassay guided fractionation of the EtOAc fraction of Terminalia phanerophlebia led to the isolation of two bioactive compounds that were identified by NMR (1D and 2D) spectroscopic methods as methyl-3,4,5-trihydroxybenzoate (1) and 1,6-di-O-coumaroyl glucopyranoside (2).

The $^1$H NMR (MeOD) of compound (2) indicated the presence of two separate AA'BB' aromatic systems, two trans-olefinic groups and sugar signals with anomeric proton at δ 5.61 (1H, d, J=7.7 Hz). The $^{13}$C and DEPT (MeOD) spectra of the compound showed seven pairs of almost identical signals assigned to the two coumaroyl groups along with six sugar peaks, Table 5.2. The positions of the attachment of two coumaroyl were assigned to C1 and C6 of the sugar using 2D NMR as indicated in Figure 5.13. Compound (2) was characterised as
1,6-di-O-coumaroyl glucopyranoside (phenyl propanoid glycoside) from its NMR spectra data in Figures 5.7 to 5.12. To the best of my knowledge, the complete NMR data of this compound is presented for the first time. However, it has previously been identified as a constituent of strawberry leaf material by semi-preparative HPLC fractionation and H NMR. This is a hyphenated technique. It does not require isolation of compound but its direct identification from the extracts (HANHINEVA et al., 2009). Characterisation of the compound was carried out using $^1$H NMR and UPLC-qTOF-MS/MS in ES (−) technique. Their structural analysis could not be achieved by data obtained from the $^1$H NMR in the study. However, structure was proposed basically from the elemental composition of the main ion (C$_{24}$H$_{24}$O$_{10}$), M/z = 471.13 and its subsequent fractionation in UPLC-qTOF-MS/MS. (HANHINEVA et al., 2009). The two compounds isolated from the EtOAc fraction are reported for the first time from *Terminalia phanerophlebia*. However, methyl-3,4,5-trihydroxybenzoate has previously been reported from *Terminalia* species such as *Terminalia chebula* Retz., *Terminalia macroptera* Guill. & Perr., *Terminalia myriocapa* Van Heurck & Müll. Arg., *Terminalia calamansai* (Blanco) Rolf., *Terminalia laxiflora*, *Terminalia bellerica* (Gaertn.) Roxb., *Terminalia horrenda* Steud. and *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn. (MOHAMED et al., 2002; CHEN et al., 2009; MAHAJAN and PAI, 2010; PHAM et al., 2011; ACHARYYA et al., 2012; EL-AMEEN et al., 2013; RASHED and ONO, 2013; PFUNDSTEIN et al., 2010).

### 5.4.2. Antimicrobial assays results

The results of the MIC values demonstrated by the fractions and isolated compounds from *Terminalia phanerophlebia* against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Mycobacterium aurum* A+ and *Mycobacterium tuberculosis* H37Ra are presented in Table 5.3. The results were interpreted as those MIC values less than 1 mg/ml had good antimicrobial activity (YORK et al., 2012). The extracts showed a broad spectrum of activity against the selected bacteria with MIC values ranging from 0.008 to >1 mg/ml. All four solvent fractions (hexane, DCM, EtOAc and butanol) obtained from the MeOH extract showed good antimicrobial activity, however, the EtOAc fraction demonstrated the lowest inhibitory values when compared to the others against all four bacterial strains tested, with MIC values ranging from 0.008 to 0.125 mg/ml. The best antimicrobial activity in this study was demonstrated by the EtOAc fraction at a MIC value of 0.008 mg/ml against...
Staphylococcus aureus. This led to the selection of the EtOAc fraction of Terminalia phanerophlebia for isolation of compounds responsible for good antimicrobial activity observed. Out of the 10 test tube fractions obtained from the EtOAc column fractionation, fractions 1 and 5 exhibited good antimicrobial activity against all four bacterial strains tested, and that led to their purification and isolation of compounds. The two isolated compounds in this study showed good antimicrobial activity against all tested bacterial strains at MIC values ranging from 0.063 to 0.250 mg/ml. Biological activity of 1,6-di-O-coumaroyl glucopyranoside (2), are reported for the first time. However, closely related compounds in the class phenylpropanoid glycoside exhibited significant antimicrobial activity against both Gram-positive and Gram-negative bacterial strains (DIDRY et al., 1999; KYRIAKOPOULOU et al., 2001; LIMA et al., 2003; SI et al., 2007). Good MIC values ranging from 0.063 to 0.125 mg/ml exhibited by 1,6-di-O-coumaroyl glucopyranoside against the tested bacterial strains were noteworthy. When compared to that of the crude extracts, 1,6-di-O-coumaroyl glucopyranoside exhibited activity which was three times better than that of the crude extracts against a strain of Mycobacterium tuberculosis. Inhibition of Mycobacterium tuberculosis by 1,6-di-O-coumaroyl glucopyranoside was noteworthy, as this bacterial strain is reported to be the leading cause of tuberculosis worldwide. Compound (2), 1,6-di-O-coumaroyl glucopyranoside could serve as a lead compound for tuberculosis drug discovery. Methyl-3,4,5-trihydroxybenzoate (1) is a biochemically available medicinally important compound derived from numerous plant species and possesses several biological activities including antibacterial, antifungal, antioxidant, antiasthmatic, anti-Herpes simplex virus and antineoplastic properties (KANG et al., 2008; CHOI et al., 2009; TEKE et al., 2011; MADIKIZELA et al., 2013b). Methyl-3,4,5-trihydroxybenzoate demonstrated high activity against Herpes simplex virus type 1 and 2 (KANE et al., 1988). This compound is considered as a useful antimicrobial agent. Therefore, the lower MIC values demonstrated by methyl-3,4,5-trihydroxybenzoate in this study were not surprising as it has demonstrated good antibacterial activities against both Gram-negative and Gram-positive bacterial strains. In in vitro studies by KANG et al. (2008), CHOI et al. (2009), and TEKE et al. (2011), methyl-3,4,5-trihydroxybenzoate demonstrated significant antimicrobial activity. KIM et al. (2008) did a study determining the effect of methyl-3,4,5-trihydroxybenzoate on intracellular survival of Mycobacterium fortuitum and Mycobacterium tuberculosis 5 h after phagocytosis, this compound significantly increased the rate of intracellular killing of both bacterial strains demonstrating significant efficacy. According to TEKE et al. (2011), the presence of
hydroxyl groups on methyl-3,4,5-trihydroxybenzoate could be responsible for the antimicrobial activity observed from this compound.

Good antimicrobial activity exhibited by the compounds isolated from *Terminalia phanerophlebia* authenticates the traditional use of this plant in treating tuberculosis and its related symptoms. Further pharmacological studies on antimicrobial evaluation of these compounds against drug-resistant strains of bacteria as well as toxicology testing are required. Antimicrobial compounds isolated from plants may have better antimicrobial properties when combined with conventional drugs, therefore, further studies on the synergy between isolated compounds and antibiotics is required to confirm if there could be any synergism between the two.
Figure 5.2(a): $^1$H NMR spectrum of Compound 1
Figure 5.2(b): $^1$H NMR spectrum of Compound 1
Figure 5.3: $^{13}$C NMR spectrum of Compound 1
Figure 5.4: DEPT NMR spectrum of Compound 1
Figure 5.5: HSQC NMR spectrum of Compound 1
Figure 5.6: HMBC NMR spectrum of Compound 1
Figure 5.7(a): $^1$H NMR spectrum of Compound 2
Figure 5.7(b): $^1$H NMR spectrum of Compound 2
Figure 5.7(c): $^1$H NMR spectrum of Compound 2
Figure 5.8(a): $^{13}$C NMR spectrum of Compound 2
Figure 5.8(b): $^{13}$C NMR spectrum of Compound 2
Figure 5.8(c): $^{13}$C NMR spectrum of Compound 2
Figure 5.9: DEPT NMR spectrum of Compound 2
Figure 5.10(a): COSY NMR spectrum of Compound 2
Figure 5.10(b): COSY NMR spectrum of Compound 2
Figure 5.11(a): HSQC NMR spectrum of Compound 2
Figure 5.11(b): HSQC NMR spectrum of Compound 2
Figure 5.11(c): HSQC NMR spectrum of Compound 2
Figure 5.12 (a): HMBC NMR spectrum of Compound 2
Figure 5.12(b): HMBC NMR spectrum of Compound 2
<table>
<thead>
<tr>
<th>No</th>
<th>(^1\text{H}) (500 MHz)</th>
<th>(^1\text{C})</th>
<th>No</th>
<th>(^1\text{H}) (500 MHz)</th>
<th>(^1\text{C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.61 (1H, d, J= 7.7 Hz)</td>
<td>95.8</td>
<td>7'</td>
<td>7.74 (1H,d, J= 15.9 Hz)</td>
<td>148.2</td>
</tr>
<tr>
<td>2</td>
<td>7.91 (3H, m, H-2, H-3, H-4)</td>
<td>74.1</td>
<td>8'</td>
<td>6.38 (1H, d, J= 15.9 Hz)</td>
<td>114.3</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>78.0</td>
<td>9'</td>
<td>C=O</td>
<td>167.7</td>
</tr>
<tr>
<td>4</td>
<td>71.5</td>
<td>1''</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.68 (1H, m,)</td>
<td>76.4</td>
<td>2'', 6''</td>
<td>7.46 (2H, d, J= 8.7 Hz)</td>
<td>131.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>4.50 (1H, dd, J= 12.1, 2.1Hz, H-6a)</td>
<td>64.5</td>
<td>3'', 5''</td>
<td>6.79 (2H, d, J=8.6 Hz)</td>
<td>117.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4.32 (1H, dd, J= 12.1, 5.6 Hz, H-6b)</td>
<td></td>
<td></td>
<td>4''</td>
<td>161.7&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>1'</td>
<td>127.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7''</td>
<td></td>
<td>7.65 (1H, d, J=15.9 Hz)</td>
<td>147.0</td>
</tr>
<tr>
<td>2', 6'</td>
<td>7.47&lt;sup&gt;a&lt;/sup&gt; (2H, d, J= 8.7 Hz)</td>
<td>131.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8''</td>
<td>6.36 (1H, d, J=15.9 Hz)</td>
<td>114.9</td>
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<tr>
<td>3', 5'</td>
<td>6.81&lt;sup&gt;b&lt;/sup&gt; (2H, d, J= 8.6 Hz)</td>
<td>117.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9''</td>
<td>C=O</td>
<td>169.3</td>
</tr>
<tr>
<td>4'</td>
<td>162.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a-f</sup> Interchangeable NMR data
Figure 5.13: Some definitive HMBC correlations of compound 2

Figure 5.14: Structures of isolated compounds from *Terminalia phanerophlebia*
Table 5.3: Antibacterial activity of the crude extracts, solvent fractions, column fractions from ethyl acetate sample and isolated compounds from the leaves of *Terminalia phanerophlebia*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacterial strains tested and minimum inhibitory concentration (MIC) values (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K. pneumoniae</em></td>
</tr>
<tr>
<td>Crude</td>
<td>0.125</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.250</td>
</tr>
<tr>
<td>Hexane</td>
<td>0.250</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.125</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.250</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>0.125</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.250</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>0.125</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>0.125</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>0.125</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>0.063</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>0.063</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>0.063</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>0.125</td>
</tr>
<tr>
<td>Fraction 10</td>
<td>0.125</td>
</tr>
<tr>
<td>Methyl-3,4,5-trihydroxybenzoate</td>
<td>0.125</td>
</tr>
<tr>
<td>1,6-di-O-coumaroylglucopyranoside</td>
<td>0.125</td>
</tr>
</tbody>
</table>

5.5. Conclusions

Investigation of the EtOAc soluble fraction of *Terminalia phanerophlebia* leaf extract using bioassay guided isolation techniques afforded a phenylpropanoid glycoside identified as 1,6-di-O-coumaroyl glucopyranoside and methyl-3,4,5-trihydroxybenzoate as its bioactive constituents. According to our knowledge, complete NMR spectra data of 1,6-di-O-coumaroyl glucopyranoside are reported for the first time. Isolated compounds are also reported for the first time from the extracts of *Terminalia phanerophlebia*. Good antimicrobial activity exhibited by these compounds validate the use of *Terminalia phanerophlebia* in traditional treatment of tuberculosis and related symptoms in South Africa. Further studies on cytotoxicity testing, combination with synthetic drugs of these isolated compounds as well as evaluating them against drug-resistant strains of bacteria are required.
Chapter 6: General conclusions

Respiratory tract infections ranging from common cold to life threatening infectious diseases such as tuberculosis are responsible for the increased mortality (7 million deaths) worldwide. Globally, the increasing resistance to the available range of drugs against pathogens causing these infections remains a major concern. South Africa is included among four countries estimated to have the leading number of XDR tuberculosis. Given the high possibility that more drug-resistant tuberculosis strains which will exhaust the current chemical defence will emerge in the future creates an urgent reason for counteracting the problem. New anti-tuberculosis agents with new modes of activity and low toxicity are required to control the epidemic of tuberculosis and reduce the prevalence of drug-resistant tuberculosis strains.

The medicinal value of plants for the treatment of various diseases and ailments was recognised by different cultures since the beginning of human civilisation. Besides consisting of great cultural diversity, South Africa is blessed with a diverse flora (more than 24 000 plant species) which has potential for the discovery of phytochemicals that have pharmacological activity. Several South African plants have been documented as exhibiting anti-tuberculosis property, therefore there is considerable potential in discovering plants that can inhibit the growth or eradicate microorganisms that cause tuberculosis. In the current study, the crude extracts from 10 plant species were tested for antimicrobial activity against bacterial strains related to respiratory ailments, and the ability to inhibit the COX enzymes. The extracts that demonstrated noteworthy antimicrobial activity (MIC values less than 1 mg/ml) were further investigated for their genotoxicity and cytotoxicity effects. Furthermore, the compounds from antimicrobial active plant extracts were isolated, identified and evaluated for antimicrobial activity.

Different solvents including PE, DCM, 80% EtOH and water were used for extraction as they cover a wide polarity range. The EtOH extract of *Polygala fruticosa* gave the highest yield, whilst PE extracts of the roots of *Terminalia phanerophlebia* yielded the lowest mass. Some of the extracts from *Abras precatorius* subp. *africanus* (leaves and seeds), *Asparagus falcatus* (leaves), *Asparagus africanus* (leaves), *Ficus sur* (roots), *Indigofera arrecta* (leaves), *Leonotis intermedia* (leaves), *Pentanisia prunelloides* (leaves and roots) and *Terminalia phanerophlebia* (leaves roots and twigs) showed noteworthy antimicrobial activity against at
least one bacterial strain or more. Thus, partially providing supporting evidence for their use in South African TM for treating tuberculosis and related symptoms. Despite being documented to be used in the treatment of tuberculosis and related symptoms, extracts of *Brunsvigia grandiflora* (bulb), *Ficus sur* (bark), *Indigofera arrecta* (roots), *Leonotis intermedia* (stem) and *Polygala fruticosa* (whole plant) did not show good antimicrobial activity.

Several symptoms of tuberculosis are associated with the host inflammatory responses that occur as a result of the immune system failing to recover. The high inhibition of COX-2 exhibited by the PE extracts of *Pentanisia prunelloides* (roots) and the DCM extracts of *Ficus sur* (bark) as well as *Abrus precatorius* subsp. *africanus* (leaves) was noteworthy. Other extracts of the selected plants which showed moderate inhibition of the COX-2 enzyme were *Asparagus africanus* (leaves), *Brunsvigia grandiflora* (bulb), *Ficus sur* (roots), *Indigofera arrecta* (roots), *Leonotis intermedia* (leaves), *Pentanisia prunelloides* (leaves), *Polygala fruticosa* (whole plant), and *Terminalia phanerophlebia* (leaves, roots and twigs). These findings were remarkable as they demonstrate that some of the plants besides having antimicrobial activity also possess anti-inflammatory property.

Although determination of the biological activities of medicinal plants is important, their safety evaluation is crucial and necessary. The genotoxicity results revealed that all the selected antimicrobial extracts were non-genotoxic against the bacterial strains tested. The non-genotoxicity effects of tested plants coupled with the noteworthy antimicrobial activity of some extracts from these plants necessitates further anti-tuberculosis research.

Extracts with noteworthy antimicrobial activity were further evaluated for their safety using the MTT assay against monkey kidney Vero cells. The highest selectivity index value (16.43) was demonstrated by the water extract of *Terminalia phanerophlebia* (leaves). Water is the commonly used solvent in the extraction of plant constituents in TM, so the high selectivity index value of the water extracts of *Terminalia phanerophlebia* (leaves) and good antimicrobial activity of the extracts from this plant part warrants further studies. *Pentanisia prunelloides* (leaf and root), *Abrus precatorius* subsp. *africanus* (leaves), and *Asparagus africanus* (leaves) extracts also demonstrated noteworthy selectivity index values which were greater than one for the biologically active extracts tested. Some of the extracts of *Abrus precatorius* subsp. *africanus, Asparagus falcatus, Leonotis intermedia, Ficus sur* and
Indigofera arrecta showed some cytotoxicity in this study, although they were non-genotoxic against Salmonella tester strains. Therefore, this suggests that these plants must be administered cautiously, and further studies using different test models are required to evaluate their cytotoxicity.

Terminalia phanerophlebia leaf extracts demonstrated considerable antimicrobial activity against tested bacterial strains known to cause respiratory ailments. Thus, it was questioned whether the antimicrobial activity could be accredited to any specific compounds in the species. Methyl-3,4,5-trihydroxybenzoate and 1,6-di-O-coumaroyl glucopyranoside were isolated from Terminalia phanerophlebia (leaf) extracts using biological assay guided fractionation. Both compounds demonstrated noteworthy antimicrobial activity which partially authenticates the traditional use of this plant in treating tuberculosis and its related symptoms.

This study provided a basic understanding of the efficacy of the selected medicinal plants in TM and management of the tuberculosis challenge. Furthermore it has confirmed the need to test these medicinal plants for more in vitro and in vivo pharmacological activities. With regards to the pharmacological results observed in this study future research should be aimed at isolating antimicrobial compounds from Abrus precatorius subsp. africanus, Asparagus africanus, Asparagus falcatus, Ficus sur, Indigofera arrecta, Leonotis intermedia, and Pentanisia prunelloides. Toxicity studies using different test batteries are required. The current findings substantiate that the pharmacologically active plants might comprise alternative future candidates for tuberculosis treatment. Besides contributing to anti-tuberculosis research, this study will contribute to sustainable utilisation of Abrus precatorius subsp. africanus, Asparagus africanus, Asparagus falcatus, Indigofera arrecta, Leonotis intermedia, Pentanisia prunelloides, and Terminalia phanerophlebia as leaves can be used rather than roots/bark/stem.
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