EVALUATION OF ISOLATES AND IDENTIFIED PHENOLICS
FROM PELARGONIUM SIDOIDES AGAINST MYCOBACTERIUM
TUBERCULOSIS, OTHER BACTERIA AND FUNGI

BY

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Submitted in partial fulfilment of the requirements for the degree
of

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June 2005
DECLARATION

I, Sannah Patience Nkami Mativandlela, hereby declare that the work on which this thesis is based, is on my work and does not contain any significant amount of unacknowledged work of others.

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Signed:........................................

Date: 2005.06.22
DEDICATION

This thesis is dedicated to Kulani Charity (Khonamanje), for her positive support when I was away for my studies
ABSTRACT

Anecdotal evidence of two South African Geranium species (*Pelargonium reniforme* and *Pelargonium sidoides*) from the United Kingdom with regard to plants being used against tuberculosis, which lacked scientific evidence’ prompted us to investigate these two plants for their antimicrobial properties. The German herbal remedy (‘Umckaloabo’) is prepared from these two plant species and is currently being sold for bronchitis.

Acetone, chloroform and ethanol extracts were investigated against three bacteria (pathogens causing bronchitis), three fungi (fungal species associated with the upper and lower respiratory tract) and *Mycobacterium tuberculosis*. This is the first report on the extracts’ activity against *Moraxella catarrhalis*, and three fungi (*Asperigilus niger*, *Rhizopus stolonifer* and *Fusarium oxysporum*). Acetone and ethanol root extracts of *P. sidoides* and its combination with *P. reniforme* exhibited activity against bacteria at 5.0 mg/ml concentration. The fungi were significantly inhibited by the acetone and ethanol extracts of *P. reniforme* and the ethanol extract of *P. sidoides* at a concentration of 5.0 mg/ml. Antituberculosis activity was observed on acetone, chloroform and ethanol root extract of *P. reniforme* and chloroform extract of *P. sidoides* at 5.0 mg/ml concentration.

The isolation and purification of compounds were attempted using two different approaches, of which the second approach resulted in isolation of **four compounds and two flavonoids**. One flavonoid (epigallocatechin) is isolated for the first time from *P. sidoides*. Laboratory investigations showed no activity of compounds isolated against *M. tuberculosis*.

As Mycobacteria are intracellular pathogens, antimycobacterial activities may be due to either direct or indirect effects. Though the compounds in this study did not show antituberculosis activity, it can be speculated that the anecdotal evidence of TB-patients could be due to their immunostimulant activity.
List of Abbreviations

AIDS    Acquired immune deficiency syndrome
ATCC    American type culture collection
CFU     Colony forming units
DMSO    Dimethyl sulphoxide
EB      Ethambutol
GI      Growth index
HIV     Human immunodeficiency virus
INH     Isoniazid
INT     2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl
MDR     Multidrug-resistant
MIC     Minimal inhibitory concentration
MRC     Medical Research Council
NMR     Nuclear magnetic resonance
PDA     Potato dextrose agar
RIF     Rifampin
SD      Standard deviation
SM      Streptomycin
TB      Tuberculosis
TLC     Thin layer chromatography
TMP     Traditional medicinal practitioners
USA     United State of America
USSR    Union of Soviet Socialist Republics
UV      Ultra violet light
WHO     World Health Organization
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Antituberculosis activity of the fractions against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method ΔGI value (mean ± SD) of the control vial was 36 ± 8.5 for the sensitive strain.

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Chapter 1

INTRODUCTION

1.1 Background

1.1.1 Utilization of herbal drugs

Plants have adapted to the diverse habitats of the world through their physical and biochemical modifications, and they have been used traditionally as a source of treatment for various ailments for thousands of years throughout the world among all human races (Ellis, 1986). Human populations have adapted largely through the generations and application of knowledge – ecological, practical and theoretical. Today traditional societies throughout the world possess a wealth of such knowledge which they have accumulated during prolonged interaction with the natural world, and which remains fundamental to their physical, spiritual and social well being (Cotton, 1996).

Because of the fear of diseases and death every cultural group has responded by developing a medicinal system and making use of natural products to cure various ailments, but where undoubtedly plants play a major role (Ellis, 1986). According to Finimh (2001), some of these traditions and medicinal practices may seem strange and magical, others appear rational and sensible, but all of them are attempts to overcome illness and suffering, and enhance quality of life.

Many traditional healers still use plants in their crude form (herbal remedies), although Western technologies have transformed some plant products in more palatable forms like tablets, capsules and syrups. Extracts from some of the medicinal plants being used by traditional healers have been found to contain properties that inhibit the growth of bacteria, viruses and other microbes (Ndubani and Hojer, 1999).
Chapter 1  

*Introduction*

The recognition and validation of traditional medicine and the search for plant derived drugs could lead to new strategies to control various diseases (Gessler *et al*., 1994). The industrial uses of medicinal plants are mainly in traditional medicines, health foods and in pharmaceuticals. The global herbal market and industry have been growing rapidly in recent years and therefore, today medicinal plants are of utmost importance (WHO, 1987). Medicinal plants have also become the focus of discovering new drugs and an integral component of research developments in the pharmaceutical industry (Rabe and Van Staden, 1997). Such research focuses on the isolation of and direct use of active medicinal constituents of plants, semi-synthetic drugs and pharmacologically active compounds. As a result the pharmacology industry has invested vast resources into screening the active constituents of medicinal plants from all over the world (Finimh, 2001; Ulubelen *et al*., 1988).

Of the 250 000 species of higher plants known to exist on earth, only a relative handful have been thoroughly studied for all aspects of their potential therapeutic value in medicine. About 25% of prescribed drugs used in modern medicine today originated from the plant kingdom (Balick, 1990). Approximately one-third of the top selling drugs in the world are natural products or their derivatives’ often with ethnopharmacological background (Table 1.1). The practice of traditional medicine is wide spread throughout the whole world. In China about 40% of the total medicinal consumption is attributed to traditional tribal medicines and it is estimated that in the mid-nineties, receipts of more than 2,5 billion US dollars ($) have resulted from the sales of herbal medicines in Thailand. The same applies to Japan where herbal medicinal preparations are more in demand than mainstream pharmaceutical products (Lemma, 1991).

Moreover, natural products are widely recognised in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. New medicines have been discovered with traditional, empirical and molecular approaches (Harvey, 1999; Strohl, 2000). The traditional approach makes use of material that has been found by trial and error over many years in different cultures and systems of medicine (Cotton, 1996).
Another approach is by bioassay-guided fractionation of plant extracts isolation of biologically active molecules. Plants investigated for their medicinal properties have yielded many useful compounds such as quinones, flavonoids, lectins, alkaloids, glycoproteins, terpenoids, coumarins, glycosides and glycones. Examples include drugs such as morphine, quinine and ephedrine that have been in widespread use for a long time and more recently derivatised synthesized compounds such as the antimalarial artemisinin. Among the successful clinical agents derived from plants are ‘benzodiazepines’ for insomnia and anxiety attacks, ‘atenolol’ for the treatment of hypertension, ‘salbutamol’ for asthma etc. (Phillipson, 2001).

1.1.2 Medicinal plant-industry in Africa

Africa is known to be a rich source of medicinal plants and some pharmaceutical agents have been produced from local plant species. In contrast with western medicine, which is technically and analytically based, traditional African medicine takes a holistic approach: good health, disease, success or misfortune are not seen as chance occurrences but are believed to arise from the actions of individuals and ancestral spirits according to the balance or imbalance between the individual and the social environment (Anyinam, 1987; WHO, 1987). Traditional African medicine is a holistic discipline involving extensive use of herbalism combined with aspects of spirituality. Traditional rural African communities have relied upon the spiritual and practical skills of the Traditional Medical Practitioners (TMP’s) whose botanical knowledge of plant species and their ecology as well as scarcity are invaluable.

In most traditional African societies there are individuals such as herbalists, spiritualists, traditional practitioners, etc. who have the responsibility of providing relief from different diseases and social problems (Duke, 1985). It is often argued that traditional healers operate close to the people and that they are very helpful in many rural communities where modern medicine is not readily available (Cunningham, 1988). In the past, traditional healers were considered to be more effective against psychosocial illness. It is now known that traditional healers use a vast array of
medicinal plants for infections such as colds, coughs and inflammatory diseases (Shale et al., 1999). Indigenous medicinal plants are used by more than 60% of South Africans in their health care needs or cultural practices (Table 1.2). Approximately 3,000 species are used by an estimated 200,000 indigenous traditional healers (Van Wyk et al., 1997). Due to urbanization, a large informal trade business has been established with medicinal plants. Unfortunately, commercial exploitation threatens to deplete plant populations, resulting in many species being considered vulnerable to extinction and being lost from their natural habitat (Mander et al., 1995). It is estimated that 70% of South Africans consult traditional healers known as ‘inyanga’. These traditional healers use many traditional medicines derived from plants for various ailments (Table 1.3). Parts of the plants can be applied directly to wounds and cuts or prepared as powders that are used like snuff or in the form of smoke or fumes, and also as infusions (tinctures).

Among a few medicinal plants being cultivated for international trade industry are Warburgia salutaris (pepperbark tree), Siphonochilus aethiopicus (African ginger), Aloe ferox (Cape aloes), Agathosma sp. (buchu), Harpagophytum procumbens DC (devil’s claw), Pelargonium sidoides DC (Umkcaloabo) and Xysmalobium undulatum (Uzara), (George, 1997) (Table 1.2). Buchu and devil’s claw are cultivated commercially, but is also harvested in the wild, which can lead to over utilization of natural habitat. Aloe ferox Miller is sustainably harvested from the wild. A large number of species containing chemical components have the potential to play a role in the medicinal market on a global scale. At present bio prospecting is done in all plants in South Africa to determine among other things its pharmaceutical potential (Mander et al., 1995).
### Table 1.1. The world’s 25 best selling pharmaceuticals in 1991 (Phillips and Drew, 1992)

<table>
<thead>
<tr>
<th>Position</th>
<th>Product</th>
<th>Therapeutic Class</th>
<th>Sales $m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ranitidine</td>
<td>H2 antagonist</td>
<td>3,032</td>
</tr>
<tr>
<td>2</td>
<td>&quot;Enalapril</td>
<td>ACE inhibitor</td>
<td>1,745</td>
</tr>
<tr>
<td>3</td>
<td>&quot;Captopril</td>
<td>ACE inhibitor</td>
<td>1,580</td>
</tr>
<tr>
<td>4</td>
<td>&quot;Diclofenac</td>
<td>NSAID</td>
<td>1,185</td>
</tr>
<tr>
<td>5</td>
<td>Atenolol</td>
<td>β-antagonist</td>
<td>1,180</td>
</tr>
<tr>
<td>6</td>
<td>Nifedipine</td>
<td>Ca$_{2+}$ antagonist</td>
<td>1,120</td>
</tr>
<tr>
<td>7</td>
<td>Cimetidine</td>
<td>H$_2$ antagonist</td>
<td>1,097</td>
</tr>
<tr>
<td>8</td>
<td>&quot;Mevinolin</td>
<td>HMGCoA-R inhibitor</td>
<td>1,090</td>
</tr>
<tr>
<td>9</td>
<td>&quot;Naproxen</td>
<td>NSAID</td>
<td>954</td>
</tr>
<tr>
<td>10</td>
<td>&quot;Cefaclor</td>
<td>β-lactam antibiotic</td>
<td>935</td>
</tr>
<tr>
<td>11</td>
<td>Diltiazem</td>
<td>Ca$_{2+}$ antagonist</td>
<td>912</td>
</tr>
<tr>
<td>12</td>
<td>Fluoxetine</td>
<td>5HT reuptake inhibitor</td>
<td>910</td>
</tr>
<tr>
<td>13</td>
<td>Ciprofloxacin</td>
<td>Quinolone</td>
<td>904</td>
</tr>
<tr>
<td>14</td>
<td>Amlodipine</td>
<td>Ca$_{2+}$</td>
<td>896</td>
</tr>
<tr>
<td>15</td>
<td>&quot;Amoxicillin/</td>
<td>β-lactam antibiotic</td>
<td>892</td>
</tr>
<tr>
<td></td>
<td>Acid/Clavulanic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Acyclovir</td>
<td>Anti-herpetic</td>
<td>887</td>
</tr>
<tr>
<td>17</td>
<td>&quot;Ceftriaxone</td>
<td>β-lactam antibiotic</td>
<td>870</td>
</tr>
<tr>
<td>18</td>
<td>Omeprazole</td>
<td>H$_{2}$ pump inhibitor</td>
<td>775</td>
</tr>
<tr>
<td>19</td>
<td>Terfenadine</td>
<td>Anti-histamine</td>
<td>768</td>
</tr>
<tr>
<td>20</td>
<td>&quot;Salbutamol</td>
<td>β$_{2}$-agonist</td>
<td>757</td>
</tr>
<tr>
<td>21</td>
<td>&quot;Cyclosporin</td>
<td>Immunosuppressive</td>
<td>695</td>
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<tr>
<td>22</td>
<td>&quot;Piroxicam</td>
<td>NSAID</td>
<td>680</td>
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<tr>
<td>23</td>
<td>Famotidine</td>
<td>H$_{2}$ antagonist</td>
<td>595</td>
</tr>
<tr>
<td>24</td>
<td>Alprazolam</td>
<td>Benzodiazepine</td>
<td>595</td>
</tr>
<tr>
<td>25</td>
<td>&quot;Oestrogens</td>
<td>HRT</td>
<td>569</td>
</tr>
</tbody>
</table>

*aNatural product derived*
Table 1.2  Selection of indigenous medicinal plants used in the South Africa  
(Mabogo, 1990)

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Popular name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agathosma betulina (Bergius) Pill.</td>
<td>Rutaceae</td>
<td>Buchu</td>
</tr>
<tr>
<td>Agathosma crenulata (L.) Pill.</td>
<td>Rutaceae</td>
<td>Buchu</td>
</tr>
<tr>
<td>Aloe ferox Millet</td>
<td>Asphodelaceae</td>
<td>Aloe</td>
</tr>
<tr>
<td>Artemisia afra Jacq. ex Wild.</td>
<td>Asteraceae</td>
<td>Wormwood</td>
</tr>
<tr>
<td>Balanite maughanii Delile</td>
<td>Balanitaceae</td>
<td>Torchwood</td>
</tr>
<tr>
<td>Bersama tysoniana Oliv</td>
<td>Melianthaceae</td>
<td>White ash</td>
</tr>
<tr>
<td>Boophane disticha (L.f.) Herbert.</td>
<td>Amaryllidaceae</td>
<td>Tumbleweed</td>
</tr>
<tr>
<td>Bowiea volubilis Harv.</td>
<td>Hyacinthaceae</td>
<td>Climbing lily</td>
</tr>
<tr>
<td>Cassine papillosa (Hochst.) Kuntze</td>
<td>Celastraceae</td>
<td>Common saffron</td>
</tr>
<tr>
<td>Clivia miniata Regel.</td>
<td>Amaryllidaceae</td>
<td>Bush lily</td>
</tr>
<tr>
<td>Cryptocarya latifolia Sond.</td>
<td>Lauraceae</td>
<td>Broad leaved quince</td>
</tr>
<tr>
<td>Curtisia dentata (Burn.f.) C.A. Smith</td>
<td>Cornaceae</td>
<td>Assegaai</td>
</tr>
<tr>
<td>Dioscorea sylvatica (Kunth) Ecklon</td>
<td>Dioscoreaceae</td>
<td>Elephant's foot</td>
</tr>
<tr>
<td>Eucomis autumnalis (Mill.) Chitt.</td>
<td>Hyacinthaceae</td>
<td>Wild pineapple</td>
</tr>
<tr>
<td>Gunnera perpensa L.</td>
<td>Gunneraceae</td>
<td>Wild rhubarb</td>
</tr>
<tr>
<td>Harpagophytum procumbens DC.</td>
<td>Pedaliaceae</td>
<td>Devil's claw</td>
</tr>
<tr>
<td>Ocotea bullata (Burchell) Baillon</td>
<td>Lauraceae</td>
<td>Stinkwood</td>
</tr>
<tr>
<td>Pelargonium sidoides DC.</td>
<td>Geraniaceae</td>
<td>Umkcaloabo</td>
</tr>
<tr>
<td>Pittosporum viridiflorum Sims</td>
<td>Pittosporaceae</td>
<td>Cheesewood</td>
</tr>
<tr>
<td>Rrapanea melanophloeos (L.) Mez</td>
<td>Myrsinaceae</td>
<td>Cape beech</td>
</tr>
<tr>
<td>Scilla natalensis Planch.</td>
<td>Hyacinthaceae</td>
<td>Blue hyacinth</td>
</tr>
<tr>
<td>Siphonochilus aethiopicus (Schweinf.) B.I. Birtt</td>
<td>Zingiberaceae</td>
<td>African ginger</td>
</tr>
<tr>
<td>Stangeria eriopus Nash</td>
<td>Stangeriaceae</td>
<td>Natal grass cycad</td>
</tr>
<tr>
<td>Warburgia salutaris (Bertol.f.) Chiov.</td>
<td>Canellaceae</td>
<td>Pepperbark tree</td>
</tr>
</tbody>
</table>
## Table 1.3  Uses of medicinal plants (Balick, 1990)

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe Vera</td>
<td>Aloe</td>
<td>Wounds, Burns, laxative</td>
</tr>
<tr>
<td>Angelica arcangelica</td>
<td>Angelica</td>
<td>Promotes menstrual flow</td>
</tr>
<tr>
<td>Piminella anisum</td>
<td>Anise</td>
<td>Promotes digestion, antispasmodic</td>
</tr>
<tr>
<td>Cinnamomum camphora</td>
<td>Camphor</td>
<td>Antiseptic, antispasmodic</td>
</tr>
<tr>
<td>Eugenia caryophyllata</td>
<td>Clove</td>
<td>Antiseptic, antibacterial, mind &amp; body stimulant</td>
</tr>
<tr>
<td>Taraxacum officinale</td>
<td>Dandelion</td>
<td>Diuretic, antibiotic, digestive</td>
</tr>
<tr>
<td>Eucalyptus globules</td>
<td>Eucalyptus</td>
<td>Antiseptic, antifungal, expectorant</td>
</tr>
<tr>
<td>Allium sativum</td>
<td>Gallic</td>
<td>Antibiotic, expels worms, antispasmodic, diaphoretic</td>
</tr>
<tr>
<td>Zingiber officinalis</td>
<td>Ginger</td>
<td>Diaphoretic, circulatory stimulant, antiseptic, coughing, anti-inflammatory</td>
</tr>
<tr>
<td>Ginkgo biloba</td>
<td>Ginkgo</td>
<td>Anti-inflammatory, antispasmodic, anti-asthmatic, anti-allergenic</td>
</tr>
<tr>
<td>Jasminum grandiflorum</td>
<td>Jasmine</td>
<td>Anti-spasmodic, aromatic</td>
</tr>
<tr>
<td>Lavandula officinalis</td>
<td>Lavender</td>
<td>Relieves muscle spasms, antiseptic, antibacterial, antidepressant</td>
</tr>
<tr>
<td>Citrus limon</td>
<td>Lemon</td>
<td>Antiseptic, anti-rheumatic, antibacterial, antioxidant, reduces fever</td>
</tr>
<tr>
<td>Commiphora molmol</td>
<td>Myrrh</td>
<td>Stimulant, antiseptic, anti-inflammatory, astrigent, antispasmodic, expectorant</td>
</tr>
<tr>
<td>Olea europaea</td>
<td>Olive</td>
<td>Digestive, diuretic, anti-inflammatory</td>
</tr>
<tr>
<td>Petroselinum crispum</td>
<td>Parsley</td>
<td>Digestive, diuretic, anti-inflammatory</td>
</tr>
<tr>
<td>Mentha piperita</td>
<td>Peppermint</td>
<td>Carminative, relieve muscle spasms, increase sweating, antiseptic</td>
</tr>
<tr>
<td>Piper nigrum</td>
<td>Pepper</td>
<td>Antibacterial, digestive, antiseptic, reduces fever</td>
</tr>
<tr>
<td>Raphanus sativus</td>
<td>Radish</td>
<td>Digestive, mild laxative</td>
</tr>
<tr>
<td>Rauwolfia serpentina</td>
<td>Rauwolfia</td>
<td>Antidepressant, lower blood pressure</td>
</tr>
<tr>
<td>Rheum palmatum</td>
<td>Rhubarb</td>
<td>Laxative, constipating, antibacterial, eases stomach pains</td>
</tr>
<tr>
<td>Rosa gallica</td>
<td>Rose</td>
<td>Aromatic, antidepressant, anti-inflammatory, sedative</td>
</tr>
<tr>
<td>Sesamum indicum</td>
<td>Sesame</td>
<td>Digestive, aromatic, antispasmodic</td>
</tr>
<tr>
<td>Malaleuca alternifolia</td>
<td>Tea Tree</td>
<td>Antiseptic, antibacterial, anti-viral, antifungal</td>
</tr>
<tr>
<td>Valeriana officinalis</td>
<td>Valerian</td>
<td>Sedative, relaxant, relieves muscle spasms, lower blood pressure</td>
</tr>
</tbody>
</table>
1.1.3 Future of traditional medicine

Despite the increasing use of medicinal plants, their future is being threatened by complacency concerning their conservation. Each year large numbers of medicinal plants are destroyed through over-exploitation by herbalist, medicinal plant traders and also through conversion of forests to agriculture (Balick, 1990). In developed countries like France, Britain, Norway, Sweden, Switzerland, Denmark, Canada, USA, China and Japan, reserves of herbs and stocks of medicinal plants are diminishing and in danger of extinction as a result of growing trade demands for cheaper health care products in preference to more expensive target-specific drugs and biopharmaceuticals. The results of over exploitation, non-sustainable harvesting techniques, loss of growth habitats and unmonitored trade of medicinal plants have put the future of traditional medicine under threat (Hoareau and Da Silva, 1999).

Scientific validation of traditional medicinal plants is essential in order to benefit humankind. Many medical scientists still find it hard to accept that natural medicines can be good as pharmaceutical therapeutics. The increase in scientific research on traditional medicine will probably change this thinking (Finimh, 2001). Herbal remedies play a fundamental role in the traditional medicine in rural areas of South Africa. More and more people utilize traditional medicine for their major primary health care needs. Today, there is growing interest in natural and traditional medicines and scientists all over the world are looking for new cures in collaboration with traditional healers (Sindambiwe et al., 1999).

1.2 Tuberculosis

1.2.1 Epidemiology

Despite all the advances in the treatment of tuberculosis (TB), this disease continues to be one of the major health problems facing mankind particularly in the developing
countries, with India accounting for nearly 30% of the global burden. According to the World Health Organisation (WHO, 1997), global tuberculosis report for 2003, the incidence rate of TB has shown a 0.4% increase per year. Tuberculosis kills approximately 2 million people each year and the global epidemic is growing and becoming more dangerous. The breakdown in health services, the spread of HIV/AIDS and the emergence of multidrug-resistant (MDR) TB are contributing to the worsening impact of this disease. In 1993, the WHO took an unprecedented step and declared tuberculosis a global emergency. It is estimated that between 2002 and 2020, approximately a billion people will be newly infected, more than 150 million people will get sick, and 36 million will die of tuberculosis. Today, *M. tuberculosis* is responsible for more morbidity in humans than any other bacterial disease. *M. tuberculosis* infects 1.7 billion people per year which is equal to 33% of the entire world population. Since 1985, the number of TB cases has risen every year. This rise is largely attributed to the emergence of HIV/ AIDS infections that has occurred during the same period of time.

TB was the cause of the “White Plague” of the 17th and 18th centuries in Europe. During this period nearly 100% of the European population was infected with *Mycobacterium tuberculosis* and 25% of all adult deaths were caused by *M. tuberculosis* (Fadda and Rowe, 1984). In the USA in Maryland, new TB cases rose from 262 in 2001 to 306 in 2002, mainly among the elderly in Montgomery, Prince George's counties and in Baltimore (Mitnick *et al.*, 2003). The national tuberculosis rate fell to a record low in 2002, according to recent statistics, in Virginia in the USA. Cases of the contagious disease continues to climb, and officials are warning that more money and stronger initiatives are needed for this kind of public health threat. The latest report by the federal Centers for Disease Control and Prevention put the county's TB rate at 9.8% cases for every 100,000 residents. That's almost twice the national rate of 5.2% increase cases and represents more active cases than in all but 14% cases in U.S. cities. Although TB cases fell in 2002 to an all-time national low in the Washington region, of 15,078 since reporting began in 1953, where cases spiked, according to the federal report released in 2003.
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The current threat in TB treatment lies on the fact of emergence of strains resistant to two most effective antituberculosis drugs, isoniazid (IND) and rifampicin (RIF). The prevalence of Multi-drug resistant TB (MDR-TB) is the highest (14.1%) in Estonia in USSR. There are a number of countries that have made remarkable progress in expanding population coverage with cure rates whereas, South Africa battles with more than 188 000 new TB cases per year (Bloom, 2002). The Medical Research Council’s National Tuberculosis Research Programme estimates that South Africa will see the number of TB sufferers approaching 300 000 in 2003. Taking into account the link between TB and HIV, it is even more disturbing to note that almost 50% of those suffering from TB will also be HIV positive. Statistics show that in the absence of HIV infection, only about 10% of TB-infected individuals get sick with TB during their lifetime. In people who are co-infected with HIV and TB, about 50% will develop TB. TB also probably accelerates the progression of the HIV (Lodha and Kabra, 2004).

1.2.2 Mycobacterium tuberculosis

*M. tuberculosis* is the etiologic agent of tuberculosis in humans. *M. tuberculosis* is a fairly large non-motile rod-shaped bacterium (Figure 1.1a). The rods are 2 – 4 μm in length and 0.2 – 0.5 μm in width. The cell wall structure of *M. tuberculosis* contains peptidoglycan and lipids which consists of major components; mycolic acids which are α-branched lipids in cell walls, make up 50% of the dry weight of the mycobacterium cell envelope and are very strong hydrophobic molecules that form a lipid shell around the organism (Goren, 1990). Cord factor is a glycolipid (trehalose dimycolate) found in the cell wall that induces replication in vitro, resulting in serpentine cords of organisms. The role of the cord in the pathogenesis of tuberculosis is still under investigation, however, it is thought to be important because it inhibits and induces secretion of TNF-alpha by macrophages (Brennan, 1988). *M. tuberculosis* is an obligate aerobe and is always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15 – 20 hours, a physiological
characteristic that may contribute to its virulence. *M. tuberculosis* is not classified as either a Gram-negative or Gram-positive bacteria because it does not have the biochemical characteristics of both. If a Gram stain is performed on *M. tuberculosis*, it stains very weakly Gram-positive or not at all. *Mycobacterium* species, along with members of a related genus *Norcardia*, are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. One acid-fast staining method for *M. tuberculosis* is staining with carbon-fuchsin (a pink dye) and decolourising with acid alcohol. The smear is counterstained with methylene blue or certain other dyes (Figure 1.1b; Fadda and Rowe, 1984; Dunigan *et al.*, 1995).

Infection with *M. tuberculosis* occurs by inhalation of small (1 - 10 microns) droplets containing only a few live tubercle bacilli. The primary focus of infection is usually therefore, the middle or lower zones of the lung. The bacilli are readily taken up by lung macrophages but survive and grow to form the primary focus of infection and from there, entering the local lymphatic and then throughout the body via the blood and lymphatic system. This stage of disease is usually clinically silent or associated with mild fever and in most cases immunity develops within a few weeks and the patient becomes tuberculin positive (Girling *et al.*, 1989).
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Figure 1.1  *M. tuberculosis*

(a) Rods of *M. tuberculosis* Magnification: x 6.250 (based on a 35 mm slide image of 24 mm in the narrow dimension (Courtesy: SEM/97229A).
(b) Colonies of *M. tuberculosis* on Lowenstein-Jensen medium

1.3 Other bacterial infections

Bacteria are the oldest, the simplest, unicellular organisms with the most numerous forms of life and are responsible for much more than just disease. Bacteria existed long time about 3.5 billion years ago, until recently, the term bacteria was used for all microscopic prokaryotes. There are thousands of different kinds of bacteria; most of them are harmless to humans. About two thousand species of bacteria have been identified; it is also possible for bacteria to reproduce as often as every twenty minutes (Crockett, 1994). A bacterium’s structure is a prokaryotic cell consisting of a capsule, cell wall, cell membrane and cytoplasm; there are no intracellular organelles. Bacteria are found almost everywhere even in the Dead Sea. They are important for their role of decomposition, leading to the release of carbon to the atmosphere that
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Plants use and also essential nutrients into the air and soil. With no carbon dioxide there would be no photosynthesis (Flannery, 1997). Some bacteria form symbiotic relationships in the gastrointestinal tract of animals as well as humans, assisting with digestion. Bacteria even produce essential vitamins, such as vitamin B12, which is essential for humans. Vitamin B12 is the most important vitamin produced by *Pseudomonas denitrificans*. Deficiency of which leads to pernicious anaemia. Other important producers are species of the genus *Propionibacterium*. The other important vitamin made commercially by micro-organisms is Riboflavin. Most riboflavin is produced by a fungus called *Ashbya gossifyii*, however, bacteria can also produce it. Doctors and scientists have figured out how to use dead or weakened bacteria to prevent other bacterial diseases (vaccination). A number of drugs, hormones or antibodies are obtained from bacteria (Schlessinger, 1990).

Bacteria are classified according to shape (bacilli, cocci and spiral), ability to form spores, nutritional requirements and reaction to the Gram stain. The Gram stain is a purple dye called crystal violet, which is washed out by the application of alcohol or acetone from Gram-negative cells. Bacteria that are not discoloured by the alcohol or acetone washed are the Gram-positive (Cullimore, 2000). Man is a host to a variety of pathogenic bacteria, protozoa and viruses. Bacteria colonize a particular part of a person's body and remain there, often without causing illness. Some may even help the body function, as in the case of intestinal bacteria. Persons who are deficient in the production of circulating antibodies are highly susceptible to respiratory infections by Gram-positive bacteria; persons who are deficient in T-cell functions, however, tend to succumb to infections by fungi and viruses, as well as to bacteria which grow predominantly intracellularly. Strep throat, cholera, pneumonia, diarrhoea and whooping cough are some of the diseases, which are caused by the Gram-positive and Gram-negative bacteria (Table 1.4; Stanier *et al*., 1976).

Respiratory infections can be divided into upper respiratory tract infections (URTI's); including tonsillitis, sinusitis and otitis media, which is the inflammation of the area behind the eardrum (tympanic membrane) producing pus, fluid, causing coughs and the lower respiratory tract infections (LRTI’s) including bronchitis and
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Pneumonia. Bacterial infections are usually preceded by viral respiratory tract infections. Lower respiratory infections are caused by secondary infections by pathogens that are inhaled from the environment, spread from blood, or breathed in from the upper respiratory tract such as from microorganisms causing bronchitis; *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* (Benjamin *et al.*, 1991).

In this study, the selected plants have been investigated for their antibacterial activity against the types of bacteria that cause acute bronchitis including *S. pneumoniae, M. catarrhalis* and *H. influenzae*.

Table 1.4. Gram-positive and Gram-negative bacteria associated with upper and lower respiratory infections

<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Sore throat, coughs</td>
</tr>
<tr>
<td><em>Enterobacter sp.</em></td>
<td>Fever</td>
</tr>
<tr>
<td><em>Haemophilus influenza</em></td>
<td>Bronchitis, pneumonia</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>Pneumonia</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>Fever, chest pains, pneumonia</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Pneumonia</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>Coughs, fever</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Nose, throat, pneumonia</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>Bronchitis, pneumonialae, ear infections,</td>
</tr>
<tr>
<td><em>Streptococcus pneumonia</em></td>
<td>Bronchitis, pneumonia, sinus and ear infections</td>
</tr>
</tbody>
</table>

1.3.1 *Haemophilus influenza*

*H. influenza* is a Gram-negative coccobacilli bacterium, found in low numbers as indigenous micro flora of the upper respiratory tract, an opportunistic pathogen causing pneumonia, epiglottitis, bronchiotitis and meningitis. This bacteria is not the cause of influenza, influenza is caused by a virus. *H. influenza* have been categorised as a, b, c, d, e and f on the basis of antigenic differences in their capsular material. The case-fatality rate for invasive *H. influenza* disease is 2% to 5%. Invasive diseases caused by *H. influenza* type b can affect many organ systems.
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The most common types of invasive diseases are bronchitis, meningitis, epiglottitis, pneumonia, arthritis, and cellulites. Type b is the one most often associated with serious diseases in children, possesses a capsule that is a polymer of ribose and ribitol phosphate (commonly called PRP). *H. influenza* is an obligate human parasite that is passed from person to person by way of the respiratory route. It is reported that 30 – 50% of all children carry the bacillus in the nasopharynx (Brook, 2002). All organisms in the genus *Haemophilus* are morphologically similar (small 0.3 x 2µm rods), though they are pleomorphic and often vary from coccoid to long, filamentous or distorted forms. They are among the most highly adapted and fragile parasitic and pathogenic bacteria with fastidious nutrient requirements. Most grow well on “chocolate agar” (i.e. infusion agar, with about 10% blood, heated to 90ºC for 10 minutes) though some require special nutrition (Fuerst, 1983).

1.3.2 *Moraxella catarrhalis*

The genus *Moraxella* is named after Morax, a famous French ophthalmologist-bacteriologist, who first isolated the type species *Moraxella laculata* (Boom *et al*., 1992). *M. catarrhalis* is a Gram-negative, aerobic oxidase + diplococcus that was described for the first time in 1886 with the name ‘*Mikrokokkus catarrhalis*’. The organism also has been known as *Micrococcus catarrhalis*, *Neisseria catarrhalis* and *Branhamella catarrhalis*. In the twentieth century, *M. catarrhalis* was considered a saprophyte of the upper respiratory tract with no significant pathogenic consequence (Karlidag, *et al*., 2002). *M. catarrhalis* causes bronchitis and pneumonia in children and adults with underlying chronic lung disease and occasionally is a cause of bacteremia or meningitis, especially in patients who are immunocompromised (Benjamin *et al*., 1991). It colonizes the throat of about 5 percent of healthy children and a higher percentage of adults with chronic lung disease.

*M. catarrhalis* has also been associated with nosocomial infections. The most significant infections caused by *M. catarrhalis* are upper respiratory tract infections (0.2 – 8.1%), including otitis media and sinusitis in children and lower respiratory
tract infections in adults. Infections with *M. catarrhalis* in adults are more common if underlying conditions are present, especially in elderly persons. (Girst *et al.*, 1979).

### 1.3.3 *Streptococcus pneumonia*

*S. pneumonia* formerly named, 'diplococcus pneumoniae', and commonly called the pneumococcus, was isolated in 1881 by Pasteur and was later shown to be the major cause of lobes of pneumoniae in humans (infecting one or more lobes of the lung). *S. pneumonia* is Gram-positive bacteria with a well-formed capsule. The organisms are characterized by a coccus appearance, a thick cell wall and aerobic action on glucose.

This bacterium is commonly found in the nose and throat. This organism is one of the commonest seen in community-acquired pneumonia, accounting for up to 25% of these infections. *S. pneumonia* has a high fatality rate, being a frequent cause of death in children and the elderly (Benjamin *et al.*, 1991). *S. pneumonia* is considered invasive when it is found in the blood, spinal fluid or other normally sterile sites. It infects the upper respiratory tract and can cause pneumonia, lining of the brain and spinal cord (meningitis), bones (osteomyelitis), joints (arthritides), ears (otitis media) and sinuses (sinusitis and bronchitis).

### 1.3.4 Gram-positive and Gram-negative bacteria

Both Gram-positive and Gram-negative bacteria contain peptidoglycan in the cell wall, but in Gram-negative, various other layers protect it and so the stain cannot reach inside to cause the colouration, the outer membrane is a lipid bilayer consisting of lipopolysaccharides. On the inside of the peptidoglycan layer is the cell membrane, which contains proteins. Additionally phospholipids, protein, lipoprotein and a small amount of peptidoglycan are present. These always face the outside and are involved in increasing the barrier for molecules entering the cell. The space between the peptidoglycan layer and the outer membrane is called the periplasm, containing many different proteins. Hydrophobic and larger molecules cannot pass through and this is
how the Gram stain is prevented from reaching the peptidoglycan layer to colour it (Ainsworth and Sussman, 1968). In Gram-positive bacteria there is a very big variation in structure and composition.

The peptidoglycan is on the outside of the Gram-positive bacteria and the stain can easily reach it. The peptidoglycans, which are sometimes also called murein, are heteropolymers of glycan strands, which are cross-linked through short peptides. There have also been some minor variations recorded in composition in some groups. Thus, in *Mycobacterium* and *Nocardia* the N-acetyl moiety of the muramic acid is replaced by the oxidised form *N*-glycolyl. The amino acid composition of both the cross-linking as well as the stem polypeptides can vary extensively with different groups. These differences form the basis for the taxonomy of these organisms (Martha *et al.*, 1997). On the side-chains are carried the bases for the somatic antigen specificity of these organisms. The chemical composition of these side chains both with respect to components as well as arrangement of the different sugars determines the nature of the somatic or antigen determinants, which are such important means of serologically classifying many Gram-negative species (Abraham *et al.*, 1993).

The difference between Gram-positive and Gram-negative bacteria is in the permeability of the cell wall to these 'purple coloured iodine-dye complexes' when treated with the decolourising solvent. The cell wall of Gram-positive bacteria, such as *B. subtilis*, *S. pyogenes* and *S. aureus*, retain gentian violet. In contrast, Gram-negative bacteria, such as *E. coli* and *V. cholerae*, which do not have a comparable cell wall, do not retain gentian violet (Cullimore, 2000).

**1.4 Fungal infections**

Fungi differ from bacteria in possessing a higher number of chromosomes within a well-defined nuclear membrane, mitochondrion, and an endoplasmic reticulum. Like plants they have definite cell walls, but these are usually composed of chitin, glucan, chitosan, mannan and other components in various combinations, rather than
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cellulose. They lack chlorophyll, so they live either on dead organic material as saprophytes, or on living organic matter as parasites. The cells may live separately (yeasts) or more commonly, they form long multicellular filaments or hyphae, which may contain cross-walls or septa (Ansteid et al., 1999). A mass of hyphae is a mycelium. Many species have both yeast and mycelial forms which are dependent on the cultural conditions, a process known as dimorphism (Alexopoulos et al., 1996). The classification of fungi is based on the form of their sexual reproductive apparatus, but there is a large group, containing most of the human parasites, which have never been known to undergo sexual reproduction (Ellis et al., 1994). The following are the main four classes of fungi:

1. **Zygote fungi (zygomycota):** There are about 600 different species of zygote fungi. Most of them are terrestrial and live in soil or decaying plant material. One of the most common types of zygote fungi is bread mould (*Rhizopus stolonifer*), some of the zygote fungi form mycorrhizae (*R. nigricans*) with plants for them to live on. *Rhizopus* soft rot is a disease caused by these types of fungi, the hyphae secrete pectinolytic enzymes that break down the middle lamellae of infected tissue and causes a soft, watery rot. The fungi lack cutinases and, therefore, can enter host tissue only through wounds.

2. **Sac fungi (ascomycota):** There are over 60 000 different species of sac fungi and they therefore have a wide range of habitats and characteristics, members of the sac fungi are important in digesting resistant materials such as cellulose and collagen. Yeasts (*Saccharomyces*), molds, morels and truffles (*Claviceps*) are examples of sac fungi. They produce sexual spore in saclike ascii and most carry out their sexual stages in a fruiting body known as an astrocarp. Asexual spores are produced in long chains or clusters at the end of the hyphae and they are dispersed with the wind. Sac fungi contain toxic alkaloids, including lysergic acid diethylamide (LSD). When infected rye is made into bread, the toxins are ingested and cause vomiting, muscle pain, feeling hot or cold, hand and foot lesions, hysteria and hallucinations.
3. Club fungi (basidiomytes): There are about 25,000 species of club fungi, most of them have a club-shaped basidium which gives them their name, for example, the mushroom, puffballs, bracket fungi, birds nest fungi, and stinkhorns. These fungi are extremely important in the decomposition of organic matter. They reproduce asexually. They cause some serious plant diseases such as rusts and smuts.

4. Imperfecti fungi (deuteromycotina): Imperfect fungi are mycelial fungi that produce by means of conidia that are generally produced on free or aggregated conidiophores on the substrate surface. These fungi lack a sexual or a perfect stage. They have lost their ability to reproduce sexually. They have developed parasexual reproduction in which clear fusion occurs but not meiosis proper.

Different organs in the human body are infected by different fungal species (Table 1.5). Systematic mycoses is a respiratory disease caused by fungal pathogens. Coccidioidomycosis is caused by *Coccidioides immitis*, infecting the pulmonary spaces. Histoplasmosis is caused by the fungus *Histoplasma capsulatum*, causing mild lung infections. Blastomycosis is caused by the fungus *Blastomyces dermatitidis* infecting the human by the airborne route. *Aspergillus, Penicillium, Cladosporium, Fusarium, Paecilomyces*, dust-borne *Zygomycetes*, and dust-borne. *Alternaria* species are often associated with lower respiratory infection, bronchitis and chronic pulmonary disease. Toxicity by inhalation can be 40 times greater than by ingestion (Kowalski and William, 1998).

**Table 1.5. Fungal pathogens associated with upper and lower respiratory infections**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Malassezia furfur</em></td>
<td>Skin</td>
</tr>
<tr>
<td><em>Microsporum</em> species</td>
<td>Skin, hair</td>
</tr>
<tr>
<td><em>Epidermophyton</em> species</td>
<td>Skin, hair, nails</td>
</tr>
<tr>
<td><em>Fusarium</em> species</td>
<td>Lungs, nose, throat</td>
</tr>
<tr>
<td><em>Penicillium</em> species</td>
<td>Lungs, sinus</td>
</tr>
<tr>
<td><em>Blastomyces dermatitidis</em></td>
<td>Lungs, ear infections, throat</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Lungs, nose, Central Nervous System</td>
</tr>
<tr>
<td><em>Aspergillus</em> species</td>
<td>Respiratory system, bone marrow failure</td>
</tr>
<tr>
<td><em>Rhizopus</em> species</td>
<td>Lungs, nails, skin, ear infections</td>
</tr>
</tbody>
</table>
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1.4.1 Aspergillus niger

The Aspergillus fungus (sac fungi), causes different types of pulmonary disease: inhalation of spores may result in bronchial asthma or acute allergic alveolitis. The fungus can colonize old tuberculous or bronchiectatic cavities, in which it may invade the lung tissue to produce a haemorrhagic and necrotizing pneumonia. The last condition tends to occur in immunodeficient or immunodepressed individuals, where the fungus may also invade the walls of the pulmonary vein, causing local thrombosis, and becoming disseminated by the blood stream: which can result in the infection of the heart valve (MacSween and Whaley, 1992).

Aspergillus species are common aspergillosis causing agents in humans and in animals. A. niger is one of the frequent agents of otomycosis and of pulmonary “fungus ball” (Ammari et al., 1993). Pulmonary cavities in the upper lobe of human lung and chronically obstructed paranasal sinuses can permit the growth of large mycelial masses, called fungus balls. In the lung, a fungus ball (1 – 2cm) may reach several centimetres in diameter. Aspergillosis is a rare complication of AIDS. It is usually seen in patients with advanced disease (Ioachim, 1989). Allergic fungal sinusitis (infection of the sinus) is a unique, probably under-diagnosed condition similar to the lower airway disorder, allergic bronchopulmonary aspergillosis (Schonheyder, et al., 1988). Characteristic features of fungal sinusitis are signs or symptoms of chronic sinusitis and infection of the lungs. Other fungal pathogens associated with this condition including Aspergillus, are the Curvularia, Drechslera, Bipolaris, Exserohilium, Alternaria, Helminthosporium and Fusarium species (Pursell, 1992).

A. fumigatus is the most common cause of all forms of aspergillosis, with A. flavus being the second, and A. niger being the third. They are probably the most important contaminants of man-made and naturally occurring organic material. They are also able to produce mycotoxins when they grow as contaminants in food. These Aspergillus species are associated with asthma, bronchitis, hypersensitivity
pneumonitis (alveolitis) and cystic fibrosis, and have been found to be dwelling in walls, carpets, mattresses and wooden window frames of some houses (Kauffman, et al., 1995).

1.4.2 *Fusarium oxysporum*

Infection caused by *F. oxysporum* (sac fungi), is called 'fusariosis', which usually occurs in immunocompromised individuals (Booth, 1971). *Fusarium* attacks cells in humans much the way it attacks cells in plants through the secretion of mycotoxins. These mycotoxins dissolve the cell walls, and the fungus is then free to absorb the cell’s contents, enter the cell cavity, reproduce and continue the process of attacking other cells. Many of these mycotoxin-producing species have estrogenic, emetic and feed refusal syndromes (Gastaldi et al., 1994). Human infection usually occurs as a result of invasion of the organisms through the body surfaces, thus causing skin infection, respiratory tract infections in patients suffering from tuberculosis, bronchitis or arthritis. *F. oxysporum* produces 70% mortality rate in immunocompromised patients (Monier et al., 1994). *F. oxysporum*-infections are rare but devastating infections caused by this common fungus. It is more commonly known as a fungus that destroys crops. However, immunocompromised patients are increasingly at risk for contracting an infection. Most of the initial infections are as a result of inhalation of the spores and involves the respiratory tract infections of the lungs (Bennett, 1995).

1.4.3 *Rhizopus stolonifer*

*Rhizopus* (zygote fungi) species, are strictly terrestrial fungi, their spores often floating around in the air and are either saprophytes or weak parasites of plants and plant products on which they cause soft rots or moulds. *Rhizopus* species may cause mucorosis in immune compromised individuals. The Zygomycetes have well-developed mycelia without cross walls and produce non-motile spores in sporangia. *Rhizopus* species are frequently found in house dust, soil, fruits, nuts and seeds.
Chapter 1

Introduction

Exposure to large numbers of *rhizopus* spores has reportedly caused respiratory complications. *Rhizopus* can be an allergen and opportunistic pathogen for immunocompromised individuals, especially those with diabetic ketoacidosis, malnutrition, and severe burns or in some cases, the common cold (Lunn, 1977). In most cases the air-borne diseases caused by *R. stolonifer* are associated with sinus problems and upper respiratory tract infections (Anstead, *et al*., 1999). The sites of infection are the lungs, nasal sinus passages, brain, eyes and skin. This mold produces mycotoxins, which can be inhaled and ingested (Campbell and Sewart, 1980).

1.5 Literature review

Over the past decade there has been a proliferation of literature on the antibacterial, antifungal and antiviral properties of plant extracts. There are several reports on *in vitro* inhibition of mycobacterium by medicinal plants. Ten of 408 ethanolic extracts of plants such as *Actaea spicata*, *Angustura vera*, *Cinnamomum camphora*, *Piper cubeba*, *Guauacum officinale*, *Ipomea purga*, *Rhamnus cathartica* inhibited growth of *M. tuberculosis* H37Rv at dilutions of 1 in 160 to 1280 and a high proportion of the other extracts inhibited growth at lower dilutions (Grange and Dawe, 1990). It was found that *M. tuberculosis* was also sensitive towards the Rwandese medicinal plants, *Pentas longifolia*, *Tetradenia riparia* and *Bidens pilosa*. The active compound isolated from the leaves of *T. riparia* was tested against *M. tuberculosis* and showed activity at 100 µg /ml (van Puyvelde *et al*., 1994). *Hydrocotyle asiaticum* inhibited growth of *M. tuberculosis* at a dilution of 1:20 (Grange and Dawe, 1990).

A number of plants have been cited in the literature as being used for medication against various bacterial and viral infections or as containing biologically active compounds. Research conducted by Noristan, Pretoria, suggests that from a total number of about 300 plants screened, at least 31% show marked analgesic, anti-inflammatory and anti-infective properties (Theunis *et al*., 1992). Out of 100 medicinal plants of Rwanda, 30% of the plants tested showed activity against *B. subtilis* and *S. aureus* (Boily and Van Puyvelde, 1986). A significant antibacterial
activity was displayed by a novel diterpene diol isolated from *Iboza riparia* (De Kimpe *et al*., 1992). The zones of inhibition produced by water and methanolic extracts of *Bridelia ferruginea* ranged from 4 to 20 mm when tested against *S. aureus*, *E. coli*, *K. pneumonia S. pyrogenes* etc. Organic extracts of *Helichrysum crispum* inhibited the growth of *M. tuberculosis* and *P. aeruginosa* (Salie *et al*., 1996).

Plant extracts from *Artemisia aucheri, A. scoparia, Carthamus oxyacantha, Francoeuria undulate, Tripleurospermum disciforme* and *Xathium spinosum* have been found to be with antifungal activity (Salehi-Surmaghi and Amin, 1993). Plants have been endowed with therapeutic virtues both in legend and in scientific literature and are being used in treating various ailments such as coughs, colds, other pathogenic bacterial and viral infections. The use of antimicrobials from natural vegetation has a great impact in human health care of undeveloped countries. Herbal medicine has been used for centuries in rural areas by local healers and has been improved in industrialized countries. A number of substances used in modern medicine for the treatment of serious diseases have originated from research on medicinal plants (Theunis *et al*., 1992).

1.6 *Pelargonium reniforme* Curtis and *Pelargonium sidoides* DC.

The traditional use of the South African plant species; *Pelargonium reniforme* Curtis and *Pelargonium sidoides* DC. in treatment of various acute and chronic infections like bronchitis, sinusitis, tonsillitis, tuberculosis and rhinopharyngitis has been reported (Watt and Breyer-Brandwyk, 1962). However, the activity of extracts has not been scientifically validated as yet against Gram-negative bacteria; *M. catarrhalis*, the fungal pathogens; *A. niger, F. oxysporum* and *R. stolonifer* and *M. tuberculosis*. 
1.7 Scope of thesis

1.7.1 Antibacterial, antifungal and antituberculosis activity of *P. reniforme* and *P. sidoides*

We intend to investigate the crude acetone and ethanol extracts of *P. sidoides* and *P. reniforme* against *H. influenza*, *M. catarrhalis*, *S. pneumonia*, *A. niger*, *F. oxysporum*, and *R. stolonifer*. Acetone, ethanol and chloroform extracts of these two plants will also be tested for activity against *M. tuberculosis*.

1.7.2 Bioassay guided fractionation of *P. sidoides*

Bioassay guided fractionation of the best extract will be conducted and an attempt will be made to isolate and purify the active principle.

1.8 Structure of thesis

Chapter 1 describes the potential of medicinal plants for various ailments.

The selection, description and phytochemical constituents of the plant species selected for the present study is reported in chapter 2.

The detection of antibacterial assay of crude extracts of *P. reniforme* and *P. sidoides* against bacteria that cause bronchitis and the other infections are described in chapter 3.

Chapter 4 reports on the antifungal activity of crude acetone and ethanol extracts of *P. reniforme* and *P. sidoides* by using the agar dilution method.
Chapter 5 reports on the antimycobacterial results of *P. reniforme* and *P. sidoides* by the BACTEC radiometric method for susceptibility testing of *M. tuberculosis*.

In Chapter 6 evaluation of different extracts from *P. sidoides* are discussed.

In Chapter 7 bioassay guided fractionation of *P. sidoides* is discussed.

In Chapter 8 evaluations of the isolated compounds from butanol extract of *P. sidoides* is discussed.

Chapter 9 comprises of general discussion and conclusion, summarizing the motives of the entire research, the importance of *P. reniforme* and *P. sidoides* as traditional medicine and recommendations from the findings.

Chapter 10 includes references.

Chapter 11 people contributing towards this project have been acknowledged.
Chapter 2

Importance of *Pelargonium reniforme* and *P. sidoides* and their phytochemical constituents

2.1 Introduction

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

Plant-derived medicine has been part of traditional health care around the world for thousands of years, and there is an increasing interest in plant sources to fight microbial diseases (Palombo and Semple, 2001). Plants contain numerous biological active compounds, many of which have been shown to have antimicrobial activity (Lopez *et al*., 2001; Karaman *et al*., 2001). The search for biologically active extracts based on traditionally used plants is still relevant due to the appearance of microbial resistance of many antibiotics and the occurrence of fatal opportunistic infections. Ethnobotanical data have proved to be useful in the search of compounds isolated from plants (Penna *et al*., 2001).
Chapter 2 Importance of P. reniforme, P. sidoides and their phytochemical constituents

2.2 Selection of plants

The selection of plants for evaluation in the present study, was based on ethnobotanical and ethnopharmacological data. The family of Geraniaceae consists of five genera; Geranium, Erodium, Monsonia, Sarcocaulon and Pelargonium. The genus Pelargonium is derived from the Greek word Pelargos, which means stork. This refers to the rostrum of the schizocarp (seed capsule).

The importance of Pelargonium species is well documented (Watt and Breyer-Brandwijk, 1962). The genus Pelargonium comprises of more than 250 natural species of perennial small shrub, which are limited in their geographical distribution. About 80% of Pelargonium species are confined to the southern parts of Africa, while others occur in Australia, New Zealand and the far East (Van der Walt and Vorster, 1988). These species usually grow in short grassland and sometimes with occasional shrubs and trees on stony soil varying from sand to clay-loam, shale or basalt. The plants are evergreen when cultivated, but die back during droughts and winter (May to August).

P. reniforme and P. sidoides are highly valued by traditional healers for their curative properties and they are well known to generations of Khoi / San and Xhosa (South African tribes) traditional healers (Wagner and Bladt, 1975). The Xhosa and the Zulu tribes of South Africa use these species to treat coughs, diarrhoea and tuberculosis (Watt and Breyer-Brandwijk, 1962). The medicinally active ingredients are found in the bitter tasting roots of the plants (Helmstader, 1996). A commonly used medicine produced in Germany, named, ‘Umckaloabo’ originates from the roots of P. sidoides and P. reniforme (Helmstader, 1996; Kolodziej and Kayser, 1998). This herbal medicine is extensively used in Germany for bronchitis, antibacterial and antifungal infections. Although this herbal medicine (Umckaloabo) is successfully employed in modern phytotherapy in Europe to cure infectious diseases of the
Chapter 2 Importance of P. reniforme, P. sidoides and their phytochemical constituents

respiratory tract, the scientific basis of its remedial effect is still unclear (Kayser and Kolodziej, 1995).

2.3 P. reniforme and P. sidoides

Figure 2.1. Plants of (a) P. reniforme and (b) P. sidoides

P. reniforme and P. sidoides belong to the family Geraniaceae, genus Pelargonium and section Reniformia (Figure 2.1a and 2.1b). The common name of P. sidoides is ‘Kalwerbossie’, which bears dark, reddish-purple (almost black) flowers (Van der Walt and Vorster, 1988). P. sidoides forms a rosette like plant with crowded leaves, it is similar to P. reniforme (Figure 2.1a), but its broad petals easily distinguish it (Figure 2.1.b). In the Republic of South Africa, P. sidoides and P. reniforme occur throughout the Eastern Cape, Free State, Southern Gauteng, South-western Gauteng and the neighbouring country of the Republic of South Africa, Lesotho (Figure 2.2). P. sidoides is found at altitudes ranging from near sea level to 2300 m in Lesotho. It is found in areas, which receive rainfall in summer (November to March) varying from
Chapter 2 Importance of P. reniforme, P. sidoides and their phytochemical constituents

200 – 800 mm per annum (Van Wyk et al., 1997). P. reniforme has dull green, small "felty" leaves with magenta flowers. It grows wild, sending out long bulbous roots deep into the ground. The species enjoys a wide reputation among the native population for its curative or palliative effects in the treatment of gastrointestinal, hepatic and respiratory tract disorders (Heil and Reitermann, 1997). P. reniforme is highly esteemed by traditional healers for its curative properties and it is well known to generations of Khoi / San and Xhosa traditional healers. Following the well-documented therapeutic use amongst the local population of South Africa, extracts of P. reniforme and P. sidoides are successfully employed in modern phytotherapy to cure infectious diseases of the respiratory tract in European countries and these form the basis of herbal medicines in areas of Southern Africa (Watt and Breyer-Brandwijk, 1962).

Figure 2.2. Distribution of P. reniforme and P. sidoides in South Africa
Chapter 2  Importance of *P. reniforme*, *P. sidoides* and their phytochemical constituents

2.4 Phytochemical constituents of *P. reniforme* and *P. sidoides* and their uses

The bioactive ingredients in both *P. sidoides* and *P. reniforme* are the tri- and tetra-oxygenated coumarins, gallic acid and gallic acid methyl ester (polyphenols) and various flavonoids (Latté *et al.*, 2000). *P. sidoides* contains two distinct coumarins: umckalin and its 7-O-methyl ester, together with four other methoxycoumarins and three unique coumarin sulphates that were not found in *P. reniforme*. The highly oxygenated coumarins, fraxinol, isofraxetin and fraxidin, together with a unique trimethoxy coumarin are found in *P. reniforme*. Scopoletin and 6,7,8-trihydroxycoumarin are found in both species (Kayser and Kolodziej, 1995). Most of the coumarins found in these two *Pelargonium* species contain a methoxy function at the C7 position and an OH group at either the C6 or C8 positions; functionality that is responsible for their antibacterial activity. Gallic acid and its methyl ester are present in large amounts in *P. sidoides* and in its active extracts, were identified as the prominent immunomodulatory principle for this herbal medicine.

Macrophage activation was reported by Kayser and Kolodziej (1997), in an *in vitro* study based on Leishmania parasites. They also studied the antibacterial performance of the various coumarins and gallic acid compounds found in *P. sidoides* and *P. reniforme* and found that with the exception of the ineffective (+) -catechin, all the compounds exhibited antibacterial activities with minimum inhibitory concentrations (MICs) ranging from of 0.2 mg/ml to 1.0 mg/ml. These results provide rational basis for the traditional use of ‘Umckaloabo’ for infections of the respiratory tract (Heil and Reitermann, 1997).
Chapter 2  Importance of *P. reniforme*, *P. sidoides* and their phytochemical constituents

2.4.1 Coumarins from *Pelargonium*

Coumarins owe their class name to “coumarou”, the vernacular name of the tonka bean, from which coumarin was isolated in 1820 (Bruneton, 1999). Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. Coumarin and other members of the coumarin family belong to benzo-α-pyrone group. Coumarin may also be found in nature in combination with sugars, as glycosides. Like other phenylpropanoids, coumarins arise from the metabolism of phenylalanine via a cinnamic acid (Bruneton, 1999; Matern et al., 1999). Coumarins are unsaturated aromatic lactones and many of them have medicinal properties. The roots of *P. reniforme* and *P. sidoides* were found to consist of distinct coumarins, such as umckalin, 7-acetoxy-5,6-dimethoxycoumarin, artein, 7-O-methyl ether, 6,8-dihydroxy-7-methoxycoumarin, 6,8-dihydroxy-5,7-tetramethoxycoumarin, three unique coumarin sulfates etc. Furthermore, the highly oxygenated coumarins; fraxinol, isofraxetin and fraxidin have been found to be associated with 8-hydroxy-5,6,7-trimethoxycoumarin as representatives of *P. reniforme* (Latté et al., 2000).

Coumarins act as vitamin K antagonists, they tend to prevent blood clotting, which prolongs bleeding time (Murray et al., 1982). Certain type of coumarins, especially 4-hydroxycoumarins (like warfarin), are used as medicines in strictly controlled dosage forms. If it is taken in relatively larger quantities it causes internal bleeding. There are some cattle poisoning cases reported in Italy from *Fercila* species due to the internal bleeding caused by 4-hydroxycoumarins. This poisoning is also known as ferulosis. Coumarins are practically found in almost every plant family. Plants use them as growth inhibitors (anti-auxins) as well as defense compounds. However, in plants of certain families such as Leguminoseae (bean family), Rutaceae (citrus family) and Umbelliferae (apiaceae) coumarins exist in larger quantities (Keating and O’Kennedy, 1997).
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2.4.2 Essential oils from Pelargonium

Essential oils are the natural essences or steam distilled from dried or wet plant material such as the roots, seeds, flowers, leaves, bark, needles, fruits and wood of plants. Methods used for extracting essential oils are, steam distillation, cold pressing, enfleurage, solvent extraction, turbodistillation, hydrodiffusion, and carbon dioxide extraction. Steam distillation is the most common method of extracting essential oils.

The plant material is placed in the plant chamber of the still, and pressurized steam is generated in a separate chamber and circulated through the plant material. The heat of the steam forces the tiny intercellular pockets that hold the essential oils to open and release them. The temperature of the steam must be high enough to open the pouches, yet not so high that it destroys the plants or fractures or burns the essential oils. As they are released, the tiny droplets of essential oil evaporate and, together with the steam molecules, travel through a tube into the still's condensation chamber.

Essential oils are mainly used for perfumery. The resulting essential oil then contains the entire aroma and the therapeutic properties of the source from which it is obtained. Pure essential oils are of high quality, unadulterated and 100% pure. Essential oils contain many organic constituents, including vitamins, hormones and other natural elements that make up the life force of plants. Due to the small quantity of these oils, they should not be used undiluted (Chaumont and Leger, 1992).

Geranium oils are obtained from several Pelargonium species but the most commonly used are the rose scented P. graviolens and P. capitatum. The essential oil accumulates in small glands found in the foliage and flowers. There are several types of geranium or essential oils namely; Reunion, Algerian, Moroccan and French. These oils are composed chiefly of geraniol, citronellol, linalool, citronellylformate and several other compounds. Reunion oil is very rich in citronellol and has a heavy rose and minty odour. Algerian oil has a delicate odour. Moroccan oil is similar to Algerian oil. French oil is thought to posses the finest rose-like odour.
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(Pattnaik et al., 1996). The essential oils of P. reniforme and P. sidoides were obtained by hydrodistillation from the leaves of the plants with 0.52% and 0.71% yields respectively. For both species, sesquiterpenes were the dominating components, with caryophyllene epoxide as the most abundant compound of the oil of P. sidoides and the sesquiterpenes hydrocarbons δ-selinene and δ–cadinene as the main constituent of the oil of P. reniforme (Kayser et al., 1998).

The oils from Pelargonium species have been described as natural perfumes. They are often used to scent soaps and detergents because, unlike many other essential oils, geranium oils are not affected by the alkaline nature of soap products. Geranium oils can range from very sweet and rosy to musty, minty and green odours. Its actions include analgesic, antiseptic, astringent, antidepressant, deodorant, insecticide and tonic. On the physiological level, geranium oil has anti-inflammatory and haemostatic properties and its use could be considered for oily complexions, menstrual problems, menopause, PMS (pre-menstrual syndrome) and haemorrhoids. Some components of the essential oil have been shown to have a stimulating effect on the pituitary gland that in turn regulates the production of the hormone oestrogen and progesterone by the ovaries (Lis-Balchin et al., 1998a).

Traditionally, geranium oil was used to help with stress-related conditions, depression, acute fear, rigidity, self-esteem, heartache, relief of fatigue, nervous tension, neuralgia, balancing emotions, for sore throat and tonsillitis, heals wounds, ulcers, skin disorders, improves circulation, cellulitis, edema, breast engorgement, as well as to treat diarrhoea, dysentery and colic. Antibacterial properties and insecticidal actions were found when geranium oil was screened for medicinal usage (Lis-Balchin, 1995).
Chapter 2 Importance of *P. reniforme*, *P. sidoides* and their phytochemical constituents

2.4.3 Flavonoids from *Pelargonium*

Flavonoids were discovered in 1936 by Scent-Gyogy from a piece of lemon peel. The term flavonoid gathers a very broad range of natural compounds belonging to the family of polyphenols. Their physical function seems to be the colouring of the plants. Flavonoids are a class of water-soluble pigments that have a carbon skeleton C₆-C₃-C₆. They originate in autotrophs but through the process of ingestion, make their way into the animal kingdom. The flavonoids occur either as aglycones of glycosides. Some classes are distributed more widely than others, most common are flavones and flavonols and the most restricted in their occurrence are isoflavones, chalcones and aurones (Aeschbacher *et al.*, 1982).

Plants could not have survived if they were not protected from the aggressions of these soluble pigments. These pigments are also used to attract pollinating insects and birds. Flavonoids have been in the different parts of the plants, especially in the aerial parts and one of them in free form called agliconas flavonoids. The anthocyanins are mainly like salts in flowers, fruits and weaves with positioning that go of red until the violet and the blue one, which is common in *Pelargonium* species (Vinson and Bose, 1988). Flavonoids have an amount of pharmacological properties attributed including enzyme activities, anti-inflammatory, antibacterial, anticancer and antiviral activities.

Flavonoids decrease the permeability of the capillary by their property “vitamin p”, they act against free radicals, collecting or destroying them. Flavonoids are also anti-allergic agents, protecting the liver against damage and antispasmodic. They decrease the cholesterol level, they are diuretic and deteriorate collagen fibres, slowing down ageing and allowing the maintenance of tissue toxicity. Anthocyanins are a class of flavonoids that are responsible for most of the red, purple and blue colour of flowers, fruits and leaves. The three most common anthocyanidins were elucidated by the German chemist Richard Willstatter around 1915. They are
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pelargoniidin from *Pelargonium* species (Peterson and Dwyer, 1998). Latté *et al.*, (2002), reported for the first time the unique series of C-2′-acylated C-glycosylflavones extended by the discovery of the C-8-glucosyl derivatives 2′-O-galloyl-vitexin and 2′-O-galloylorientin and their C-6 analogues 2′-O-galloylosovitexin and 2′-O-galloylisoorientin, representing the first described O-galloyl-C-glycosylflavones associated with non-galloylated parent analogues and the flavonoid pattern of the roots and aerial parts of *P. reniforme*.

2.4.4 Tannins from *Pelargonium*

The word tannin is very old and reflects a traditional technology. “Tannin” (waterproofing and preserving) was the word used to describe the process of transforming animal hides into leather by using plant extracts from different plant parts of different plant species. Tannins are mainly located in the vacuoles or surface wax of the plants; they are common both in Gymnosperms and Angiosperms. Tannins belong to the group of simple and complex phenol, polyphenol and flavonoid compounds bound with starches and often so amorphous that they are classified as tannins simply because at some point in degradation they are astringent and contain variations of gallic acid. Tannins do not interfere with plant metabolism, they are generally protective substances found in the outer and inner tissues. Tannins are oligomeric compounds with multiple structure units with free phenolic groups. They are soluble in water, with exception of some high molecular weight structures. They have the ability to bind to proteins to form insoluble or soluble tannin-protein complexes (Haslam, 1998).

The two main categories of tannins that impact on animal nutrition are hydrolyzable tannins (HT) and condensed tannins identified more correctly as proanthocynidins. The hydrolysable tannins are molecules with polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or
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totally esterified with phenolic groups like gallic acid derived from quinic acid (gallotannins) or ellagic acid (ellagitannins). Ellagitannins are formed from hexahydroxydiphenic acid by the oxidative coupling of neighbouring gallic acid units attached to a D-glucose core. The ellagitannins are present in most Pelargonium species (Giner-Chavez, 1996).

Five new ellagitannins from the aerial parts of P. reniforme have been identified. They have been designated as pelargoniins A-D, isocorilagin, and the new phyllanthusiin, E methyl ester. These ellagitannins are accompanied by two known structurally related metabolites, corilagin and phyllanthusiin C, two phenolcarboxylic acids, brevifolincarboxylic acid and phyllanthusiin E, the gallotannin 1-O-galloyl-beta-D-glucopyranose and the ellagitannins strictinin and isostricinin having a 4C1-glucose core (Latté and Kolodziej, 2000a).

The different biological activities of tannins are due to different chemical structures. They become less astringent and reactive towards proteins, their solubility decreases and eventually they become bonded to cell wall components. The binding of tannins with proteins participates in the antidiarrhoeic activity of tannins by protecting the digestive organs from injurious attack. It also participates in the antihaemorrhagic effects of tannin rich drugs. The detoxification of snake venoms and bacterial toxins by persimmon tannin are another activity most probably based on the strong binding activity of tannins (Asquith and Butler, 1986). Tannins at relatively high concentrations usually inhibit the activity of enzymes, but at low concentrations, they often stimulate enzyme activity (Maxson et al., 1973). Tannins have been shown to inhibit the growth of HIV and herpex simples virus (Okuda et al., 1991).
Chapter 3

Antibacterial activity of *Pelargonium reniforme* and *Pelargonium sidoides*

3.1 Introduction

Plants have always been a common source of medications, either in the form of traditional preparation or as pure active principles. One of the major issues regarding developing new drugs from plants is to examine the uses claimed in traditional use. Many reports on the pharmacological testing of crude extracts have been published by investigators (Farnsworth *et al.*, 1985).

*P. reniforme* and *P. sidoides*, plant species of the Geraniaceae family, possess medicinal properties because roots are used for chest complaints, bronchitis, diarrhoea, etc. by indigenous people of South Africa (Kayser and Kolodziej, 1997). Roots of these two species have been used by the Xhosa and the Zulu tribes of South Africa for coughs, diarrhoea and tuberculosis (Watt and Breyer-Brandwijk, 1962). Other *Pelargonium* species such as *P. erodium*, *P. zonale*, *P. suburbanum*, *P. tetragonium* and *P. odorantissimum* have been cited in the literature for having antibacterial, antimicrobial, anti-inflammatory, anticoagulant, antidepressant and antiseptic properties (Lis-Balchin, 1995).

In the present study, the antibacterial properties of roots of *P. reniforme* and *P. sidoides* have been scientifically validated.
3.2 Materials and methods

3.2.1 Plant material

Roots of *P. reniforme* and *P. sidoides* were collected from Qwaqwa, a region in the Free State province of South Africa. Voucher specimens of *P. reniforme* (P 092558) and *P. sidoides* (P 092559) were deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), Pretoria.

3.2.2 Preparation of plant extracts

Dried and powdered roots of *P. reniforme* and *P. sidoides* (300g) were extracted three times with 2 litres of acetone and ethanol separately. The extracts were filtered and concentrated with rotary vacuum evaporator to dryness at reduced pressure. Acetone and ethanol extracts were reconstituted in acetone to a concentration of 50.0 mg/ml (Figure 3.1).

![Crude extracts of *P. reniforme* and *P. sidoides*](image)

**Figure 3.1** Crude extracts of *P. reniforme* and *P. sidoides*
3.3 Bacteria

Bacteria used in this investigation, *H. influenza*, *M. catarrhalis*, and *S. pneumonia* were obtained from the Department of Pathology, University of Pretoria and maintained on Colombian agar.

3.4 Antibacterial assay

For the antibacterial assay, the minimum inhibitory concentration (MIC) of the acetone and ethanol extracts were determined by incorporating various amounts (5.0, 1.0 and 0.5 mg/ml) of the extracts into chocolate agar in sterile bottles and placed in a water bath to prevent solidification, then withdrawn into petri dishes and left to solidify for approximately four hours. The bacterial colonies were transferred into sterile screw capped round tubes to which 5 ml of diluting fluid (saline) was added to achieve McFarland no.1 turbidity standard. Each suspension was streaked on petri dishes containing the extracts and the chocolate agar (Figure 3.2). The plates (three replications) were incubated at 25 °C for 24 hours and antimicrobial activity was evaluated thereafter. Streptomycin sulphate added to chocolate agar at final concentrations of 0.5, 0.01 and 0.05 mg/ml served as positive control, and three petri dishes containing 500µl acetone mixed with chocolate agar served as negative controls.

Figure 3.2  Petri dishes with extracts and chocolate agar
3.5 Results and Discussion

Of the six plant extracts tested against *H. influenza*, *M. catarrhalis* and *S. pneumonia*, it was found that the ethanol and acetone extracts of *P. sidoides* and its combination with *P. reniforme* were active at 5.0 mg/ml against *H. influenza*, *M. catarrhalis* and *S. pneumonia* (Table 3.1; Figure 3.3).

There have been a few reports of these bacterial organisms being susceptible to other plant extracts. Christoph *et al.*, 2001 found antibacterial activity of Australian tea tree oil from *Melaleuca alternifolia* Cheel and niaouli oil isolated from *M. quinquenervia* at 0.01 (% v/v) against *M. catarrhalis*. Dorman and Deans (2000), tested essential oils of *P. graveolens* and found not active against *Moraxella* sp. Crude acetone and ethanol extracts of *P. reniforme* was not active against these bacteria in contrast to the findings of Kayser and Kolodziej (1997), who found inhibition of *S. pneumonia* and *H. influenza* at concentrations of 7.5 and 5.0 mg/ml respectively by acetone extracts of roots of *P. reniforme*.

Gram-negative bacteria have been found to be less susceptible to plant extracts in earlier studies done by other researchers (Kuhnt *et al.*, 1994; Afolayan and Meyer, 1995). Similar to our results with regard to *P. reniforme* (extracts of *P. reniforme* were not active against tested bacteria). Magama *et al.*, 2002 also did not observe any antibacterial activity when methanol crude extracts and fractionated extracts (hexane, ethyl acetate, ethanol and butanol) of *Euclea crispa* were tested against *H. influenza* and *S. pneumonia* at concentration ranging from 7.0 – 0.5 mg/ml.
Chapter 3  

Antibacterial activity of *P. reniforme* and *P. sidoides*

![Image of bacterial inhibition with labels: Control, Bacterial inhibition by acetone extract of *H. influenza* and *P. reniforme*]

**Figure 3.3**  Antibacterial activity of acetone extract of *P. reniforme*
Chapter 3  

Antibacterial activity of *P. reniforme* and *P. sidoides*

### Table 3.1  
Antibacterial activity of extracts of *P. reniforme* and *P. sidoides* against *H. influenza*, *M. catarrhalis* and *S. pneumonia*

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC(^a) (mg/ml) of samples against <em>H. influenza</em>, <em>M. catarrhalis</em> and <em>S. pneumonia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pelargonium reniforme</em> (acetone)</td>
<td>5.0 (N(^b))</td>
</tr>
<tr>
<td><em>P. reniforme</em> (ethanol)</td>
<td>5.0 (N)</td>
</tr>
<tr>
<td><em>P. sidoides</em> (acetone)</td>
<td>5.0 (S(^c))</td>
</tr>
<tr>
<td><em>P. sidoides</em> (ethanol)</td>
<td>5.0 (S)</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (acetone)</td>
<td>5.0 (S)</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (ethanol)</td>
<td>5.0 (S)</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>0.01 (S)</td>
</tr>
</tbody>
</table>

\(^a\) Minimum inhibitory concentration.  
\(^b\) Not active at the highest concentration tested.  
\(^c\) Susceptible.

### 3.6 Conclusion

The bacteria inhibited in this study have been associated with infections of the respiratory tract, which the local inhabitants of South Africa treat using *P. sidoides* and *P. reniforme*. Also, the fact that the ethanol extract of *P. sidoides* and its combination with *P. reniforme* inhibited *H. influenza*, *M. catarrhalis* and *S. pneumonia*, which cause bronchitis, provides some scientific rationale for the use of the extracts for bronchitis and chronic asthma.
Chapter 4

Antifungal activity of *Pelargonium reniforme* and *Pelargonium sidoides*

4.1 Introduction

The organisms of the fungal lineage include mushrooms,rusts,smuts,puffballs,truffles,morels,molds,and yeasts,as well as many less well-known organisms (Alexopoulos *et al.*, 1996). About 70,000 species of fungi have been described; however, it is estimated that 1.5 million species may exist (Hawksworth, 1991; Hawksworth *et al.*, 1995). Fungi are found mainly in air,dust,soil,plants,and decaying organic matter. They adhere to dust particles,inhaled and deposited on the nasal-pananasal sinus mucosa. The warm,mot environment of the upper respiratory tract is an ideal environment for the proliferation of these organisms. However,they are rarely pathogenic because host resistance is high,except under favourable growth conditions in highly immunocompromised individuals.

Fungi are closely related to bacteria. They possess a unique property called dimorphism,meaning that they may exist both as a spore form and as a branching,mycelial form,depending on environmental conditions. The presence or absence of segmentations or septa of the hyphae often distinguishes the species. The hyphae branch like a tree from a central stem or from a common node such as observed in case of *Rhizopus*. The terminal buds may exhibit spherical sporangia or are arrayed in clusters like the conidiophores of *Aspergillus*. These fungi grow best on Sabouraud's agar. They are,however,difficult to grow and it may take weeks to produce identifiable colonies.
Chapter 4  

Antifungal activity of *P. reniforme* and *P. sidoides*

The most commonly seen mycotic organisms in the Western world are *Alternaria*, *Aspergillus*, *Fusarium*, *Mucor* and *Rhizopus* (Cooke and Whipps, 1993).

In this chapter, extracts of *P. reniforme* and *P. sidoides* are evaluated against fungal pathogens associated with infections of the respiratory tract system.

4.2 Materials and methods

4.2.1 Preparation of plant extracts

Acetone and ethanol extracts of the powdered roots of *P. reniforme* and *P. sidoides* were prepared as mentioned in chapter 3, section 3.2.2. Acetone and ethanol extracts were dissolved in acetone to a concentration of 100.0 mg/ml for antifungal bioassay.

4.3 Fungi

Fungal pathogens used in the study, *A. niger* (UPFC 13), *F. oxysporum* (UPFC 97) and *R. stolonifer* (UPFC 312) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each fungus was maintained on Potato Dextrose Agar (PDA) at ± 25 °C.

4.4 Antifungal assay

For the antifungal assay, the required amount of acetone and ethanol extracts were added to sterile PDA in 5 ml Petri dishes before congealing to yield final concentrations of 5.0, 1.0 and 0.5 mg/ml. PDA plates with acetone and fungi served as controls. Once the agar had solidified, a 5 mm plug of a seven-day old fungal culture was placed in the center of the petri dish containing the extract-amended and unamended PDA plates.
The plates were sealed with parafilm and placed in a 25 °C incubator. Fungal growth was measured on two diametric lines after 3, 6 and 9 days of growth. Each treatment was replicated three times. Results were statistically analyzed using student t-test.

4.5 Results and Discussion

The results of the three-day growth of tested fungi were statistically analyzed using two-way analysis of variance (ANOVA) and least significant differences (P = 0.05) were determined according to the student t-test (Table 4.1). The acetone and ethanol root extracts of *P. reniforme* and ethanol root extract of *P. sidoides*, significantly inhibited the fungal pathogens tested at a concentration of 5.0 mg/ml (Figure 4.1 a,b,d and Table 4.1). Our results are in agreement with other researchers, where Latté and Kolodziej (2000b), found that the aqueous acetone extracts of the roots of *P. reniforme* exhibited a minimum inhibitory concentration (MIC) of 8.0 mg/ml against *Asperigillus fumigatus*, *Rhizopus nigricans*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans* and opportunistic yeasts tested. All samples had no activity against the filamentous fungi at concentrations of 1.0 mg/ml.

Other plant extracts have been found to be antifungal against the fungi tested in this study. Chandrasekaran and Venkatesalu (2004), investigated the water and methanol extracts of *Syzygium jambolanum* for antifungal activity against *A. niger* and *R. stolonifer* and the highest zones of inhibition were recorded at 1.0 and 0.5 mg/ml respectively. Chamundeeswari *et al.*, 2004 found an MIC of 2.5 mg/ml when ethanol root extract of *Trewia polycarpa* was tested against *A. niger*. 

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## Antifungal activity of *P. reniforme* and *P. sidoides*

### Table 4.1  Antifungal growth on exposure to acetone and ethanol root extracts of *P. reniforme* and *P. sidoides*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration mg/ml</th>
<th><em>A. niger</em> Growth ($^a$) mm</th>
<th><em>F. oxysporum</em> Growth mm</th>
<th><em>R. stolonifer</em> Growth mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$^b$</td>
<td></td>
<td>52.0 ± 9.9</td>
<td>51.3 ± 0.3</td>
<td>23.7 ± 0.8</td>
</tr>
<tr>
<td><em>P. reniforme</em> (acetone)</td>
<td>5.0</td>
<td>3.5 ± 0.9</td>
<td>28.0 ± 0.7</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>18.75 ± 0.4</td>
<td>41.25 ± 3.9</td>
<td>25.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>42.25 ± 0.4</td>
<td>30.5 ± 0.0</td>
<td>16.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. reniforme</em> (ethanol)</td>
<td>5.0</td>
<td>0.0 ± 0.0</td>
<td>18.33 ± 1.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>28.67 ± 2.5</td>
<td>31.75 ± 1.1</td>
<td>25.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>36.33 ± 1.5</td>
<td>37.5 ± 0.7</td>
<td>16.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. sidoides</em> (acetone)</td>
<td>5.0</td>
<td>39.67 ± 5.0</td>
<td>36.0 ± 0.9</td>
<td>13.33 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>35.0 ± 3.1</td>
<td>38.33 ± 0.9</td>
<td>36.33 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>52.25 ± 0.4</td>
<td>47.33 ± 4.0</td>
<td>46.33 ± 1.5</td>
</tr>
<tr>
<td><em>P. sidoides</em> (ethanol)</td>
<td>5.0</td>
<td>0.0 ± 0.0</td>
<td>13.33 ± 0.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>35.0 ± 1.7</td>
<td>36.33 ± 0.3</td>
<td>17.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>53.67 ± 4.9</td>
<td>44.33 ± 1.5</td>
<td>42.5 ± 0.5</td>
</tr>
</tbody>
</table>

$^a$ Growth of fungal species after 72 hours (mean ± standard deviation).

$^b$ Acetone control.
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Antifungal activity of P. reniforme and P. sidoides

4.6 Fungal inhibition

(a)

(b)
Chapter 4  

*Antifungal activity of P. reniforme and P. sidoides*

Figure 4.1. Antifungal activity of:

(a) *P. reniforme* acetone extract

(b) *P. reniforme* ethanol extract

(c) *P. sidoides* acetone extract

(d) *P. sidoides* ethanol extract
Chapter 4  Antifungal activity of P. reniforme and P. sidoides

4.6 Conclusion

The fungi inhibited in this study have been associated with infections of the respiratory tract, which the local inhabitants of South Africa treat using roots of *P. sidoides* and *P. reniforme*. Also, the results obtained in this study justify the traditional usage of the two *Pelargonium* species for lung infections.
Chapter 5

Antituberculosis activity of *Pelargonium reniforme* and *Pelargonium sidoides*

5.1 Introduction

Tuberculosis is a disease of global importance and one of the most common infectious diseases known to man. One third of the world’s population is estimated to have been infected with *M. tuberculosis* and eight million new cases of tuberculosis arise each year. The tuberculosis crisis is likely to escalate since the human immunodeficiency virus (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases. The majority of tuberculosis patients are aged between 15 to 45 years, persons in their most productive years of life. Tuberculosis kills over two million people world-wide each year, more than any other single infectious diseases, including AIDS and malaria (Girling, 1989).

Transmission of tuberculosis is virtually entirely by droplet, created through coughing by untreated persons suffering from pulmonary tuberculosis (the most common form) in a confined environment. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible person (Fadda and Rowe, 1984). Different parts of the plants, such as roots are used by indigenous people to treat respiratory tract infections such as TB symptoms, colds, diarrhoea, coughs, etc. (Helmstäder, 1996). The selection of these plants was based on the actual information from published sources and experience of patients, for example from Mr. Charles Stevens, Mr and Mrs Phillip Learney, who used the plant species for treating themselves when they had TB (personal communication).
Chapter 5  

Antituberculosis activity of P. reniforme and P. sidoides

5.2 Materials and methods

5.2.1 Preparation of plant extracts

Dried and powdered roots and shoots of P. reniforme and P. sidoides were extracted with acetone, chloroform and ethanol separately. Chloroform (solvent of medium polarity) was added for antituberculosis study, because our focus was mainly to analyse efficacy of the two Pelargonium species against tuberculosis. The extracts were filtered and concentrated with rotary vacuum evaporator to dryness at reduced pressure. For antituberculosis assay, all three extracts were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 500.0 mg/ml.

5.2.2 Mycobacterium tuberculosis

A drug-susceptible strain of M. tuberculosis, H37Rv obtained from American Type, MD, USA Culture Collection (ATCC), 27294, was used to investigate the activity of the plant extracts.

5.2.2.1 Antituberculosis assay

The introduction of radiometric techniques in the field of mycobacteriology is a relatively recent development. Radiometric respiratory with the BACTEC TB-460 system apparatus (Figure 5.1), is a well-documented technique for susceptibility testing of M. tuberculosis as described previously (Lall and Meyer, 2001; Siddiqi et al., 1981). Solutions of all the extracts were prepared in DMSO to obtain a concentration of 500 mg/ml and stored at 4°C until used. Subsequent dilutions were made in DMSO and added to BACTEC 12B vials containing 4 ml of 7H12 medium broth to achieve the desired final concentrations of 5.0, 2.5, 1.0 and 0.5 mg/ml together with PANTA (Becton...
Dickinson & Company, Ferndale, South Africa), an antimicrobial supplement. Control experiments showed that the final amount of DMSO (1%) in the medium had no effect on the growth of *M. tuberculosis*. Anti-TB drugs: streptomycin (4 µg/ml), isoniazid (2 µg/ml), rifampicin (0.2 µg/ml) and ethambutanol (6 µg/ml) (Sigma Chemical Co., South Africa), were also tested against the H37Rv strain of *M. tuberculosis* which served as positive drug controls. A homogenous culture (0.1 ml) of all the strains of *M. tuberculosis*, yielding 1 x 10^4 to 1 x 10^5 colony-forming units per millilitre (CFU/ml), was inoculated in the vials containing the extracts as well as in the control vials (Heifets et al., 1985; Youmans et al., 1948). Three extract-free vials were used as controls (medium + 1% DMSO): two vials (V1) were inoculated in the same way as the vials containing the extracts, and one (V2) was inoculated with a 1:100 dilution of the inoculum (1: 100 control) to produce an initial concentration representing 1% of the bacterial population (1 x 10^2 to 1 x 10^3 CFU/ml). The MIC was defined as the lowest concentration of the extract that inhibited > 99% of the bacterial population.

When mycobacterium grows in 7H12 medium containing 14 C-labelled substrate (palmitic acid), they use the substrate and ^14^CO_2 is produced. The amount of ^14^CO_2 detected reflects the rate and amount of growth occurring in the sealed vial, and is expressed in terms of the growth index (GI) (Middlebrook et al., 1977; Snider et al., 1981). Inoculated bottles were incubated at 37°C and each bottle was assayed every day to measure GI, at about the same hour(s) until cumulative results were interpretable. The difference in the GI values of the last two days is designated as ΔGI. The GI readings of the vials containing the test extracts were compared with the control vials (V2). Readings were taken until the control vials, containing a 100 times lower dilution of the inoculum than the test vials, reached a GI of 30 or more. If the ΔGI values of the vials containing the test extracts were less than the control vials, the population was reported to be susceptible to the compound. Each test was replicated three times. Whenever results suggested contamination (e.g., large, rapid increase in GI), bottles were inspected and the organisms were stained by Ziehl-Neelsen stain to determine whether the visible microbial
growth was a mycobacterium (Kleeberg et al., 1980). With this stain, the bacilli appear as brilliantly stained red rods against a deep sky-blue background. Organisms often have a beaded appearance because of their polyphosphate content and unstained vacuoles (Joklik et al., 1968; Figure 5.2).

Since anecdotal evidence suggests the use of a combination of ethanol extracts of two Pelargoniums, we also included the combination of ethanol extracts of both the roots for antimicrobial assay.

Figure 5.1 BACTEC TB- 460 instrument
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Antituberculosis activity of *P. reniforme* and *P. sidoides*

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

- **Ziehl-Neelsen carbolfuchin**

  **Fuchin**
  
  Basic fuchin  
  3.0 g
  95% ethanol  
  100 ml
  Basic fuchin was dissolved in ethanol  
  Solution 1

  **Phenol**
  
  Phenol crystals  
  5.0 g
  Distilled water  
  100 ml
  Phenol crystals were dissolved in distilled water  
  Solution 2

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

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

  Concentrated hydrochloric acid  
  3 ml
  95% ethanol  
  97 ml
  Concentrated hydrochloric acid was carefully added to 95% ethanol.

- **Counterstain: Methylene blue**

  Methylene blue chloride  
  0.3 g
  Distilled water  
  100 ml
  Methylene blue chloride was dissolved in distilled water.
Chapter 5  

Antituberculosis activity of P. reniforme and P. sidoides

Figure 5.2  Ziehl-Neelsen staining (Kleeberg et al. 1980; WHO/ TB/97.258)
Chapter 5  

Antituberculosis activity of P. reniforme and P. sidoides

5.3 Results and Discussion

5.3.1 Inhibitory activity of plant extracts

The antituberculosis assay of extracts was interpreted on day five or six when the control vials (V2) reached a GI value of 30 or more. Acetone, chloroform and ethanol extracts from the roots and shoots of P. reniforme showed inhibitory activity at 5.0 mg/ml against the drug-sensitive strain of M. tuberculosis, but the combination of root extracts from both Pelargoniums did not show any synergy (Tables 5.1 and 5.2). Activity of the standard antituberculosis drugs used as positive controls, were much stronger than those of the extracts. Our results are in agreement with previous reports on antituberculosis activity of water extracts of Thymus vulgaris, Nidorella anomala, Cryptocarya latifolia and acetone extract of Rapanea melanophloeos where MIC’s were also found to be 5.0 mg/ml against M. tuberculosis (Lall and Meyer, 1999).

Extracts of P. sidoides were not active against M. tuberculosis similar to the results obtained by Fabry et al., 1998 where methanol extracts of Entada abyssinica, Terminalia spinosa, Harrisonia abyssinica, Ximenia caffra, Azadirachta indica and Spilanthes mauritiana were found to be inactive against M. tuberculosis at concentrations ranging from 2.0 – 0.5 mg/ml. In conclusion, we can say that the sensitivity of the extracts of the two Pelargoniums used in this study is specific to the type of bacterial organism. M. tuberculosis was only found to be susceptible to the extracts of P. reniforme and other bacteria (H. influenza, M. catarrhalis and S. pneumonia) were found to be susceptible to P. sidoides.
Chapter 5  

Antituberculosis activity of *P. reniforme* and *P. sidoides*

Table 5.1  Antituberculosis activity of the root extracts against the sensitive strain (H37Rv) of *Mycobacterium tuberculosis* as determined by the radiometric method. ΔGI value (mean ± SD) of the control vial was 20 ± 1.4 for the sensitive strain

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC(^a) (mg/ml)</th>
<th>ΔGI(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>20 ± 1.4</td>
</tr>
<tr>
<td><em>Pelargonium reniforme</em> (chloroform)</td>
<td>5.0 (S(^c))</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. reniforme</em> (chloroform)</td>
<td>5.0 (S)</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td><em>P. reniforme</em> (ethanol)</td>
<td>5.0 (S)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (acetone)</td>
<td>5.0 (S)</td>
<td>-1 ± 0.8</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (chloroform)</td>
<td>5.0 (S)</td>
<td>1 ± 0.0</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (ethanol)</td>
<td>5.0 (S)</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td><em>P. sidoides</em> (acetone)</td>
<td>5.0 (N(^d))</td>
<td>35.5 ± 6.3</td>
</tr>
<tr>
<td><em>P. sidoides</em> (chloroform)</td>
<td>5.0 (S)</td>
<td>18.5 ± 4.94</td>
</tr>
<tr>
<td><em>P. sidoides</em> (ethanol)</td>
<td>5.0 (N)</td>
<td>276 ± 9.89</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.004 (S)</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>0.006 (S)</td>
<td>0.33 ± 0.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.0002 (S)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.002 (S)</td>
<td>4.0 ± 0.0</td>
</tr>
</tbody>
</table>

\(^a\) Minimum inhibitory concentration.

\(^b\) Growth Index.

\(^c\) Susceptible.

\(^d\) Not active at highest concentration tested.
Chapter 5  

Antituberculosis activity of *P. reniforme* and *P. sidoides*

Table 5.2  Antituberculosis activity of the shoot extracts against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method. $\Delta$GIvalue (mean ± SD) of the control vial was 20 ± 1.4 for the sensitive strain

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (mg/ml)</th>
<th>$\Delta$GI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>20 ± 1.4</td>
</tr>
<tr>
<td><em>Pelargonium reniforme</em> (chloroform)</td>
<td>5.0 (S&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td><em>P. reniforme</em> (chloroform)</td>
<td>5.0 (S)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. reniforme</em> (ethanol)</td>
<td>5.0 (S)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (acetone)</td>
<td>5.0 (S)</td>
<td>-1 ± 2.8</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (chloroform)</td>
<td>5.0 (S)</td>
<td>1 ± 2.8</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (ethanol)</td>
<td>5.0 (S)</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td><em>P. sidoides</em> (acetone)</td>
<td>5.0 (N&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>28.0 ± 5.6</td>
</tr>
<tr>
<td><em>P. sidoides</em> (chloroform)</td>
<td>5.0 (N)</td>
<td>22.5 ± 12.0</td>
</tr>
<tr>
<td><em>P. sidoides</em> (ethanol)</td>
<td>5.0 (N)</td>
<td>36 ± 18.3</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.004</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>0.006</td>
<td>0.33 ± 0.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.0002</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.002</td>
<td>4.0 ± 0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Minimum inhibitory concentration.

<sup>b</sup> Growth Index.

<sup>c</sup> Susceptible.

<sup>d</sup> Not active at highest concentration tested.
5.4 Conclusion

Acetone, chloroform and ethanol extracts of *P. reniforme* showed inhibitory activity against a sensitive strain of *M. tuberculosis* (MIC 5.0 mg/ml), but the combination of extracts of *P. reniforme* and *P. sidoides* did not show any synergy. The results provide scientific rationale for the use of the extracts for tuberculosis to a certain extent.

However, on the receipt of extensive anecdotal evidences one would expect very good activity of these plants against *M. tuberculosis*. It could be speculated that the extracts and or compounds purified from extracts could be active intracellularly, in real physiological conditions similar to one of the potent antituberculosis drug ‘Pyrazinamide’. Pyrazinamide’s *in vitro* MIC against a drug sensitive strain is 0.4 µg/ml, but this compound is highly active in macrophages (Sbarbaro *et al.*, 1996).
Chapter 6

Evaluation of different extracts from *Pelargonium sidoides*

6.1 Introduction

As mentioned on Chapter 2, the plants belonging to the genus *Pelargonium* have yielded different secondary metabolites, e.g. coumarins (Figure 6.1 (a – e), and tannins (Figure 6.2), in addition to glycosides. Many of these compounds are known to possess medicinal properties.

Latté *et al.*, (2000), described the isolation of coumarins, such as umckalin, 7-O-methyl ether, 7-acetoxy-5,6-dimethoxycoumarin, 6,8-dihydroxy-7-methoxycoumarin, 6,8-dihydroxy-5,7-tetramethoxycoumarin, artelin and three unique coumarin sulfates from *P. sidoides*. Furthermore, the highly oxygenated coumarins; fraxinol, isofraxetin and fraxidin were found to be associated with 8-hydroxy-5,6,7-trimethoxycoumarin as representatives of *P. reniforme*. Kayser and Kolodziej (1997), investigated the highly oxygenated coumarins, fraxinol, isofraxetin and fraxidin, together with a unique trimethoxy coumarin found in *P. reniforme* for antibacterial activity. Scopoletin and 6,7,8-trihydroxycoumarin are found in both species. Most of the coumarins found in these two *Pelargonium* species contain a methoxy function at the C7 position and an OH group at either the C6 or C8 positions; functionality that is responsible for their antibacterial activity. Gallic acid and its methyl ester present in large amounts in *P. sidoides* and in its active extracts, were identified as the prominent immunomodulatory principle for this herbal medicine umckaloabo (Latté *et al.*, 2000). The German chemist
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Richard Willstatter, elucidated pelargoniidin from *Pelargonium* species in 1915. Latté *et al.*, 2002, first described O-galloyl-C-glycosylflavones associated with non-galloylated parent analogues and the flavonoid pattern of the roots and aerial parts of *P. reniforme* and isolated the unique series of C-2"-acylated C-glycosylflavones extended by the discovery of the C-8-glucosyl derivatives 2"-O-galloyl-vitexin and 2"-O-galloylorientin and their C-6 analogues 2"-O-galloylisovitexin and 2"-O-galloylisoorientin.

1. R = CH₃ (5,6,7-Trimethoxy coumarin)
2. R = H (6-Hydroxy-5,7-dimethoxy coumarin, umckalin)
3. R = COCH₃ (7-Acetoxy-5,6-dimethoxy coumarin)

4. R = CH₃ (6,8-Dihydroxy-7-methoxy coumarin)
5. R = H (6,7,8-Trihydroxy coumarin)
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6. \( R = \text{CH}_3 \) (5,6,7,8-Tetramethoxy coumarin, artelin)

7. \( R = \text{H} \) (6,8-Dihydroxy-5,7-dimethoxy coumarin)

8. 7-Hydroxy-6-methoxy coumarin, (scopoletin)

9. 5,7,8-Trihydroxy coumarin

**Figure 6.1** Coumarins isolated from *P. sidoides*
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**Evaluation of different extracts from P. sidoides**

In our investigation, the roots of *P. sidoides* were found to have antimicrobial activity against Gram-negative bacterial species *H. influenza*, *M. catarrhalis* and *S. pneumonia* and fungal pathogens *A. niger*, *F. oxysporum* and *R. stolonifer*. It has been documented in a book review that ‘Umckalin’, a coumarin is responsible for antituberculosis activity (Bladt, 1977).

Since bioactive compounds such as coumarins, flavonoids, tannins, etc. have been found to be present in the roots of both *P. reniforme* and *P. sidoides*, an attempt was made to isolate the active compounds from the roots of *P. sidoides*. Bioassay guided fractionation of the roots of *P. sidoides* was done using five Gram-positive, four Gram-negative bacterial species and *M. tuberculosis*. 

**Figure 6.2  Tannins isolated from *P. reniforme***

![Tannins isolated from *P. reniforme*](image)
6.2 Materials and Methods

6.2.1 Preparation of plant extracts

Dried and powdered roots of *P. sidoides* (500g) were extracted with 100% chloroform, and 70% acetone. The aqueous acetonic extract was sequentially extracted with ethyl acetate and butanol. The fractions were filtered and concentrated with rotary evaporator to dryness at reduced pressure. All fractions were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100.0 mg/ml.

6.3 Bacteria

Nine bacterial species (Table 6.1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar slant, and was recovered for testing by growing them in nutrient broth (No. 2, Biolab) for 48 hours at 37 °C. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth (Dilika and Meyer, 1996; Lall and Meyer, 2000). H37Rv, American Type Culture Collection, MD, USA 27294 experimental strain of *M. tuberculosis*, was used to investigate the activity of the plant extracts and bacterial inoculum was prepared as mentioned in Chapter 5, section 5.3.

6.4 Antibacterial assay

The antibacterial assay activity of chloroform, acetone, ethyl acetate, butanol and water fractions against Gram-positive and Gram-negative bacteria were determined by incorporating various amounts of the extracts into petri dishes containing the culture media. Different fractions of the plant extract were introduced by adding autoclaved
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nutrient agar (BIOLAB), swirled carefully until the agar began to set into sterile petri dishes. The organisms were streaked in radial patterns on agar plates containing plant extracts in laminar flow cabinet (Figure 6.3), petri dishes were sealed, incubated at 37 °C and observed after 24 hours (Mitscher et al., 1972). The fractions were tested at 5.0; 2.5; 1.0 and 0.5 mg/ml concentrations. Two blank petri dishes containing only nutrient agar and two containing nutrient agar and 1% acetone without plant extracts served as controls. In addition plates containing streptomycin sulfate at concentration of 200, 100, 50 and 10 µg/ml served as positive controls. The MIC values were regarded as the lowest concentrations of the extracts that did not permit any visible growth after 24 hour of incubation at 37 °C (Mitscher et al., 1972). Each treatment was replicated three times.

Figure 6.3  Bacterial streaking in laminar flow cabinet
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**Evaluation of different extracts from P. sidoides**

### 6.5 Antituberculosis assay

Chloroform, acetone, ethyl acetate, butanol and water fractions of the roots of *P. sidoides* were investigated for antimycobacterial activity at concentrations ranging from 5.0 to 2.5 mg/ml as described in Chapter 5, section 5.2.2.1.

### 6.6 Results and Discussion

#### 6.6.1 Antibacterial and antituberculosis bioassays of extracts of *P. sidoides*

The antibacterial bioassays of extracts of *P. sidoides* against the Gram-positive and Gram-negative bacteria species showed that butanol extract was the most significant one as compared to other extracts, inhibiting the growth of *B. cereus, B. pumilus, B. subtilis, S. aureus* and *E. coli* at concentrations ranging from 1.0 mg/ml to 2.5 mg/ml. In addition, ethyl acetate extract was found to be active at 1.0 to 5.0 mg/ml against five Gram-positive bacteria (*B. cereus; B. pumilus; B. subtilis; S. aureus and E. faecalis*) and one Gram-negative bacteria, (*P. aeruginosa*) (Table 6.1). The acetonic fraction was found to be active only against *B. cereus* at 0.5 mg/ml. Chloroform and water fractions inhibited only a few Gram-positive bacteria at concentration ranging from 1.0 to 5.0 mg/ml. The reference antibiotic, streptomycin sulfate inhibited the growth of all the bacterial species tested in this study at 0.01 mg/ml except *Pseudomonas aeruginosa* and *Serratia marcescens* which were inhibited at 0.05 and 0.1 mg/ml respectively. The antituberculosis assay of extracts was interpreted on day five or six when the control vials (V2) reached a GI value of 30 or more. Chloroform, ethyl acetate and butanol fractions from the roots of *P. sidoides* showed inhibitory activity at 5.0 and 2.5 mg/ml against the drug-sensitive strain of *M. tuberculosis*. The acetone and water fractions did not show
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inhibition on *M. tuberculosis* (Table 6.2). Similar to the results obtained by Fabry et al., 1998 where methanol extracts of *Entada abyssinica*, *Terminalia spinosa*, *Harrisonia abyssinica*, *Ximenia caffra*, *Azadirachta indica* and *Spilanthes mauritiana* were found to be inactive against *M. tuberculosis* at concentrations ranging from 2.0 – 0.5 mg/ml.

Kayser and Kolodziej (1997), tested acetone root extract at concentrations ranging from 5.0 to 7.5 mg/ml. All extracts exhibited a fairly high antibacterial effect against the spectrum of microorganisms. The highest activities consistently resided in the water extracts, with pronounced effects against *E. coli*, *K. pneumonia*, β–hemolytic *streptococcus*, *S. aureus*, *P. aeruginosa* and *P. mirabilis* with minimum inhibitory concentration (MIC) of 0.62 mg/ml and 1.25 mg/ml for *S. pneumonia* and *H. influenza*.

In our study, the ethyl acetate and butanol extracts were found to exhibit similar, but less antibacterial potencies at MIC’s of 1.0 and 2.0 mg/ml, respectively. However, various other species of *Pelargoniums* such as *P. tomentosum*, *P. odoratissium*, *P. denticulatum* have been found to possess good antibacterial activity against Gram-positive bacterial species such as *S. aureus*, *P. vulgaris*, *B. cereus*, and *S. epidermidis* at the concentration of 1.25 mg/ml (Lis-Balchin et al., 1998a). In another study Lis-Balchin et al., 1998b, found that the essential oil from *P. filcifolium* had good inhibitory activity against *Listeria innocua*, a Gram-positive bacteria.

In this study, the Gram-positive and *M. tuberculosis* bacteria appeared to be more susceptible to the inhibitory effect of extracts of ethyl acetate and butanol fractions of *P. sidoides* than the Gram-negative bacteria. The negative results obtained against Gram-negative bacteria were not surprising as, in general, these bacteria are more resistant than Gram-positive ones (Rabe and van Staden, 1997). The greater resistance of Gram-negative bacteria to plant extracts has been documented previously. Previous studies suggested that the difference in the cell wall structure between Gram-positive and Gram-negative bacteria might be the reason. The Gram-negative bacteria have an outer
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**Evaluation of different extracts from P. sidoides**

membrane acting as a barrier to many environmental substances, including antibiotics (Palombo and Semple, 2001). Accordingly, our findings slightly corresponded to the previous reports by Kayser and Kolodziej (1997) on antibacterial properties found on the fractionated ethyl acetate and butanol root fractions of *P. sidoides*; exhibiting an MIC of 1.25 mg/ml against same bacteria tested, *E. coli* and *S. aureus*. Similar observations were made by Kuhnt et al., (1994), Afolayan and Meyer (1995); Lall and Meyer (2000) and Saxena et al., (1996), while studying the antibacterial activity of *Hyptis verticillata*, *Helichrysum aureonites*, *Euclea natalensis* and *Masdevallia uniflora*.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Gram + / -</th>
<th>Samples</th>
<th>CHCl₃</th>
<th>Me₂CO</th>
<th>EtOAc</th>
<th>BuOH</th>
<th>H₂O</th>
<th>Streptomycin Sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>1.0</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus pumilus</em></td>
<td>+</td>
<td>5.0</td>
<td>na</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>+</td>
<td>1.0</td>
<td>na</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td>na</td>
<td>na</td>
<td>5.0</td>
<td>2.5</td>
<td>1.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>+</td>
<td>1.0</td>
<td>na</td>
<td>2.5</td>
<td>na</td>
<td>1.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>-</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>1.0</td>
<td>na</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-</td>
<td>na</td>
<td>na</td>
<td>2.5</td>
<td>na</td>
<td>na</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>-</td>
<td>na</td>
<td>na</td>
<td>5.0</td>
<td>na</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Minimum inhibitory concentration.

*b* Not active at the highest concentration tested.
Table 6.2  Antituberculosis activity of the root extracts against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method. ΔGI value (mean ± SD) of the control vial was 20 ± 1.4 for the sensitive strain

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (mg/ml)</th>
<th>ΔGI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pelargonium sidoides</em> (chloroform)</td>
<td>5.0 (S&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td><em>P. sidoides</em> (70% acetone)</td>
<td>5.0 (N&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>37.5 ± 7.4</td>
</tr>
<tr>
<td><em>P. sidoides</em> (ethyl acetate)</td>
<td>2.5 (S)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>P. sidoides</em> (butanol)</td>
<td>2.5 (S)</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td><em>P. sidoides</em> (water)</td>
<td>5.0 (N)</td>
<td>376.0 ± 9.97</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.004 (S)</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>0.006 (S)</td>
<td>0.33 ± 0.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.0002 (S)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.002 (S)</td>
<td>4.0 ± 0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Minimum inhibitory concentration.

<sup>b</sup> Growth Index.

<sup>c</sup> Susceptible.

<sup>d</sup> Not active at highest concentration tested.
Chapter 6  \textit{Evaluation of different extracts from \textit{P. sidoides}}

6.7 Conclusion

The Gram-positive bacteria (\textit{B. cereus}; \textit{B. pumilus}; \textit{B. subtilis}; \textit{S. aureus} and \textit{E. faecalis}) appeared to be more susceptible to the extracts obtained from \textit{Pelargonium sidoides} than the Gram-negative ones. Chloroform, ethyl acetate and butanol extracts of \textit{P. sidoides} were found to be active against \textit{M. tuberculosis} at 5.0 mg/ml.

Coumarins that have been isolated from the butanol extract of \textit{P. sidoides} previously by other researchers were found to have antibacterial properties (Kayser and Kolodziej, 1997). An attempt was therefore made to isolate compounds from the butanol extract of the plant. A number of different chromatography techniques were used, but we could not succeed to purify compounds from this extract. Due to the lack of enough plant material, further analysis with respect to purification and identification of pure compounds was carried out using a different approach from the one used as mentioned in this chapter.
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Bioassay guided fractionation of *Pelargonium sidoides*

7.1 Introduction

Plants belonging to the genus *Pelargonium* have yielded essential oils, tannins, flavonoids, phenols and coumarins. When it comes to the economic importance of these plants, some of the species have aromatic oils that are used in perfumes, cosmetics, and as insect repellant. The oil of geranium, widely used in perfumery and cosmetics, is stable and blends well with other fragrances. Dried leaves are used in sachets and potpourris (Simon *et al*., 1984). Leaves of geranium are also used in herbal teas and the oil is used in baked goods and fruit desserts. The geranium of florists comes from many annual and perennial geranium species that vary in fragrance, growth habit and leaf and flower color. The scented geraniums are extensively used in flower gardens and as potted herbs (Lis-Balchin, 2002).

Kayser and Kolodziej (1995), reported the isolation of highly oxygenated coumarins from acetone root extracts of *P. sidoides*. Latté *et al*., (2000), analysed and compared the coumarin patterns of *P. sidoides* and *P. reniforme*, forming the origin of the herbal medicine ‘umckaloabo’. In the search of the underlying active principle(s) of medicinally used *Pelargonium* species for the treatment of respiratory infections, the antibacterial activity of *P. sidoides* and *P. reniforme* constituents were evaluated by Kayser and Kolodziej (1995), followed by a systematic study on the antifungal effects of tannins and related compounds by Latté and Kolodziej (2000b).
In this study, we report the bioassay-guided fractionation of *P. sidoides* and the isolation of compounds. ‘Umckaloabo’ has been reported to be used to treat tuberculosis. However, the active principles of *P. sidoides* have not been scientifically validated for their cure against tuberculosis. In this chapter, we have investigated the efficiency of isolated compounds from *P. sidoides* against *M. tuberculosis*.

### 7.2 Materials and Methods

#### 7.2.1 Preparation of plant extracts

Fresh roots of *P. sidoides* (1 kg) were extracted with 100% ethanol. The extract was filtered and concentrated with rotary evaporator to dryness at reduced pressure and dissolved in methanol. Aqueous methanol extract was introduced to Di-ion column using water and acetone as eluent.

Fractions obtained were combined using 100% acetone and 75% acetone and sequentially extracted with ethyl acetate and butanol. Antituberculosis of all the extracts was conducted against *M. tuberculosis* as mentioned in Chapter 5, section 5.2.2.1.

#### 7.2.2 Sephadex LH-20 and silica gel column separation

Since the butanol extract showed antituberculosis activity against *M. tuberculosis* (Table 7.1), therefore, it was selected for further work. Schematic representation of the purification steps for the isolation of the compounds is illustrated in figure 7.1. The butanol fraction (15 g) was dissolved in methanol and subjected to a sephadex column using methanol as eluant (Figure 7.2). The fractions (500 ml) were spotted on TLC plates of silica gel 60 F$_{254}$ using 10% methanol in ethyl acetate (10 mL) and hexane: ethyl acetate (7:3) as eluents and analysed under UV spectrum (Figure 7.3). Similar fractions
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Bioassay guided fractionation of P. sidoides

were pooled together and dried, which resulted in three main fractions. The content of the combined dried fractions (Figure 7.1) were subjected to a sephadex column eluted with 100% methanol. Similar fractions were combined and concentrated to dryness, which resulted in four fractions. Fractions (Figure 7.1) were subjected to silica gel column eluted with chloroform and methanol in order of increasing polarity (5 – 50%). Similar fractions were combined together based on TLC profile.

Fractions II b12 (Figure 7.1) were subjected to preparative TLC of silica gel and eluted with hexane: ethyl acetate (7:3). The semipure and pure compounds (Figre 7.4), were scraped off from the preparative plate and eluted with ethyl acetate, filtered and concentrated to dryness. The samples were then sent to NMR for analysis.
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Bioassay guided fractionation of P. sidoides

Butanol crude extract (P. sidoides)
15g
Sephadex column
Methanol
66 fractions
I II III
Sephadex column
Methanol
52 fractions
II a II b II c II d
Silica column
methanol : chloroform gradient
18 fractions
II b1 II b2 II b3 II b4 II b5
Silica column
hexane: ethyl acetate gradient
13 fractions
II b11 II b12 II b13 II b14 II b15
Preparative TLC plate
4 coumarins 2 flavonoids

Figure 7.1 Schematic representation of the purification steps for the isolation of the compounds from the roots of P. sidoides
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Bioassay guided fractionation of *P. sidoides*

Figure 7.2  Sephadex column chromatography of butanol fraction obtained from the fresh roots of *P. sidoides*
Figure 7.3  TLC plates of fractions obtained from chromatographic separation of butanol extract of *P. sidoides*

Solvent systems: (a) hexane: ethyl acetate (7:3)
   (b) 10% methanol in ethyl acetate

Detection : Vanillin in H$_2$SO$_4$
Figure 7.4 Pure and semipure compounds obtained from *P. sidoides*

Solvent systems: 10% methanol in ethyl acetate

Detection: Vanillin in H$_2$SO$_4$
7.3 Results and Discussion

7.3.1. Antituberculosis results

The antituberculosis assay of extracts was interpreted on day five or six when the control vials (V2) reached a GI value of 30 or more. Ethanol and butanol extracts from roots of *P. sidoides* showed inhibitory activity at 2.5 mg/ml against *M. tuberculosis*. The fractions obtained from the butanol extract (100, 75, 50 and 25% acetone), did not show inhibition on *M. tuberculosis*. Activity of the standard antituberculosis drug (isoniazid), used as a positive control was stronger than those of the extracts and fractions (Table 7.1). Our results are in agreement with previous experiments on ethanol extract (Chapter 5, section 5.3.1), and butanol extract (Chapter 6, section 6.6.1).
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Bioassay guided fractionation of P. sidoides

Table 7.1  Antituberculosis activity of the fractions obtained from the chromatographic separation of butanol extract of Pelargonium sidoides against the sensitive strain (H37Rv) of M. tuberculosis as determined by the radiometric method. ΔGI value (mean ± SD) of the control vial was 36 ± 8.5 for the sensitive strain

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC(^a) (mg/ml)</th>
<th>ΔGI(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonium sidoides (ethanol)</td>
<td>2.5 (S(^c))</td>
<td>34.0 ± 5.7</td>
</tr>
<tr>
<td>P. sidoides (ethyl acetate)</td>
<td>na(^d)</td>
<td>40.5 ± 14.8</td>
</tr>
<tr>
<td>P. sidoides (butanol)</td>
<td>2.5 (S)</td>
<td>5.5 ± 2.1</td>
</tr>
<tr>
<td>P. sidoides (100% acetone)</td>
<td>na</td>
<td>165 ± 46.6</td>
</tr>
<tr>
<td>P. sidoides (75% acetone)</td>
<td>na</td>
<td>199.5 ± 30.4</td>
</tr>
<tr>
<td>P. sidoides (50% acetone)</td>
<td>na</td>
<td>322.5 ± 77.0</td>
</tr>
<tr>
<td>P. sidoides (25% acetone)</td>
<td>na</td>
<td>375.5 ± 51.6</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.002 (S)</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

\(^a\) Minimum inhibitory concentration.  
\(^b\) Growth Index.  
\(^c\) Susceptible.  
\(^d\) Not active at highest concentration tested (2.5 mg/ml).
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Bioassay guided fractionation of P. sidoides

7.3.2 Identification of the isolated compounds

The compounds were identified as coumarins (umckalin, scopoletin, 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one and 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one; and flavonoids (catechin and epigallocatechin), (Figure 7.5) by comparing their $^1$H-NMR and $^{13}$C-NMR spectral data (Figure 7.6 and 7.7) with the published article of highly oxygenated coumarins (Kayser and Kolodziej, 1995). Coumarins constitute a major category of secondary metabolites that are widely distributed in the plant kingdom. They are characterized by a variety of oxygenated patterns on the benzopyrone nucleus and display an array of biochemical and pharmacological actions (Murray, 1982).

Previous researchers such as Kayser and Kolodziej revealed the presence of coumarins in P. reniforme and P. sidoides. The popular herbal medicine, Umckaloabo originates from these two plant species (Kayser and Kolodziej, 1998).
Figure 7.5  Coumarins and flavonoids isolated from butanol extract of *P. sidoides*

(a) 6-Hydroxy-5,7-dimethoxy-2H-benzopyran-2-one (umckalin)
(b) 7-Hdroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)
(c) 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one
(d) 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one
(e) Epigallocatechin
(f) Catechin
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Bioassay guided fractionation of *P. sidoides*

(a) 6-Hydroxy-5, 7-dimethoxy-2H-benzopyran-2-one (umckalin)

(b) 7-Hydroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)
Chapter 7

Bioassay guided fractionation of *P. sidoides*

(c) **6.8-Dihydroxy-5, 7-dimethoxy-2H-benzopyran-2-one**

(d) **6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one**
Figure 7.6  $^1$H-NMR spectrums of coumarins and flavonoids isolated from butanol extract of $P.$ sidoides
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Bioassay guided fractionation of *P. sidoides*

(a) 6-Hydroxy-5, 7-dimethoxy-2H-benzopyran-2-one (umckalin)

(b) 7-Hydroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)
Chapter 7  

Bioassay guided fractionation of P. sidoides

(c) 6,8-Dihydroxy-5, 7-dimethoxy-2H-benzopyran-2-one

(d) 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one
Figure 7.7 $^{13}$C-NMR spectrums of coumarins and flavonoids isolated from butanol extract of *P. sidoides*
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Bioassay guided fractionation of *P. sidoides*

### 7.4 Conclusion

Bioassay guided isolation of bioactive butanol extract of *P. sidoides* resulted in the isolation of six compounds: 6-Hydroxy-5,7-dimethoxy-2H-benzopyran-2-one, 7-Hydroxy-6-methoxy-2H-benzopyran-2-one, 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one, 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one, epigallocatechin and catechin. All compounds isolated in this study have been reported from this plant previously except **compound 6 (epigallocatechin)** is reported here for the first time.
Chapter 8

Evaluations of the isolated compounds

8.1 Introduction

As a medicinal plant, Pelargonium species have traditionally been considered an astringent and used as a folk remedy in the treatment of ulcers. A terpine hydrate synthesized from geraniol is known to be, an effective expectorant. Leaves are reported to have antifungal activity. Scented geranium and oil of geranium are reported to cause contact dermatitis. Geranium is reported to repel insects because of its citronellol content (Lis-Balchin, 2002).

In this study, antituberculosis activity of the isolated compounds from fractions of butanol extract of the roots of \textit{P. sidoides} is reported.

8.2 Bioassay on \textit{M. tuberculosis}

The radiometric respiratory technique with the BACTEC apparatus was used for susceptibility testing of \textit{M. tuberculosis}. Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A drug-susceptible strain of \textit{M. tuberculosis}, H37Rv obtained from American Type, MD.USA Culture Collection (ATCC), 27294, was used in the screening procedure. Umckalin, scopoletin, 6,8-Dihydroxy-5, 7-dimethoxy-2H-benzopyran-2-one, 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one, epigallocatechin and catechin were each dissolved at 10 mg/ml in DMSO and added to 4mL of BACTEC 12B broth to achieve the final concentrations of 100, 50, 10, 5 and 1.0 µg/ml in triplicates, one with PANTA, two
without PANTA (Becton Dickinson & Company, an antimicrobial supplement). The BACTEC drug susceptibility testing was also done for the primary drug isoniazid at concentration of 0.2 µg/ml respectively against the H37Rv sensitive strain. Preparation of bacterial cultures and the testing procedures were the same as described in Chapter 5, section 5.4.1.

8.3 Results and Discussion

In the present study, fractions and compounds isolated were investigated for antituberculosis activity. Ethanol and butanol fractions from the roots of *P. sidoides* showed inhibitory activity at 2.5 mg/ml against the drug-sensitive strain of *M. tuberculosis*. The ethyl acetate did not show inhibition on *M. tuberculosis*. Coumarins and flavonoids isolated did not show activity at the highest concentration tested (Table 8.1).

Similar results were obtained by researchers; Kolodziej *et al.*, (2003), where inhibition was found on the aqueous acetone root extract of *P. sidoides* against *M. tuberculosis* at a concentration of 12.5 µg/ml, however none of the isolated phenolic compounds or coumarins showed antimycobacterial activity *in vitro*. Scopoletin, umckalin, 5,6,7-trimethoxycoumarin, (+)-catechin, gallic acid and its methyl ester and 6,8-dihydroxy-5,7-dimethoxycoumarin) in both species of *P. reniforme* and *P. sidoides* were evaluated against eight micro organisms including three Gram-positive (*S. aureus*, *S. pneumonia* and β-hemolytic streptococcus) and five Gram-negative (*E. coli*, *K. pneumonia*, *P. mirabilis*, *P. aeruginosa*, *H. influenza*) at MICs of 5 to 7.5 mg/ml. All compounds (scopoletin, 5,6,7- trimethoxycoumarin, gallic acid, gallic acid methyl ester, (+)-catechin and 6,8-dihydroxycoumarin), exhibited more or less pronounced antibacterial activities against Gram-positive and Gram-negative pathogens with the MICs of 0.2 to 2.0 mg/ml. Umckalin and 6,8-dihydroxy-5,7-dimethoxycoumarin showed antibacterial activity with MICs of 0.3 to 0.5 mg/ml. These observations by us and
Kolodziej et al., (2003), on antimycobacterial activity suggest that the coumarins are not active against *M. tuberculosis*.

### Table 8.1  Antituberculosis activity of the compounds against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method.

ΔGI value (mean ± SD) of the control vial was 36 ± 8.5 for the sensitive strain.

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>ΔGI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Hydroxy-5, 7-dimethoxy-2H-benzopyran-2-one (umckalin)</td>
<td>na&lt;sup&gt;c&lt;/sup&gt;</td>
<td>178.5 ± 55.9</td>
</tr>
<tr>
<td>7-Hydroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)</td>
<td>na</td>
<td>227.0 ± 31.1</td>
</tr>
<tr>
<td>6,8-Dihydroxy-5, 7-dimethoxy-2H-benzopyran-2-one</td>
<td>na</td>
<td>149.5 ± 70.0</td>
</tr>
<tr>
<td>6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one</td>
<td>na</td>
<td>16.0 ± 14.1</td>
</tr>
<tr>
<td>Catechin</td>
<td>na</td>
<td>146.0 ± 12.7</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>na</td>
<td>145.5 ± 12.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.2 (S&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Minimum inhibitory concentration.

<sup>b</sup> Growth Index.

<sup>c</sup> Not active at highest concentration tested (100µg/ml).

<sup>d</sup> Susceptible.
Chapter 8  
Evaluations of the isolated compounds

8.4 Conclusion

The coumarins and flavonoids isolated from the roots of *P. sidoides* did not show inhibitory activity against *M. tuberculosis*. As *Mycobacteria* are intracellular pathogens, antimycobacterial activities reported by anecdotal evidences may be due to either direct or indirect effects. In recent studies, chemical constituents and pharmacological studies have demonstrated antibacterial and immunomodulatory activities *in vitro* (Kolodziej *et al.*, 2003). Though the compounds in our study did not show antituberculosis activity, it can be speculated that the anecdotal evidence of tuberculosis-patients could be due to an immunostimulant.

The isolated compounds from *P. sidoides* have not been investigated as yet for intracellular TB activity. It is therefore, recommended that these compounds should be analysed for intracellular activity against *M. tuberculosis* in mice and / or human macrophages.
Chapter 9

General Discussion and Conclusion

Medicinal plants represent an almost unexplored reservoir of new substances with potentially useful properties. Indigenous knowledge systems have historically benefited pharmacological research programmes, which deals with future discovery of new drugs (Cox, 1990). Today we still rely on the curative properties of plants for about 75% of our medicines. It has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medicines (Prozesky et al., 2001). In many countries traditional healers, medical doctors and University researchers explore indigenous medicines to record information on the use of medicinal plants, their preparation method, to discover new botanical resources and to identify the constituents responsible for the therapeutic or toxic effects of medicinal plants. Most species of higher plants have never been investigated for their chemical or biologically active constituents, and many interesting bioactive molecules have been discovered (Aquino et al., 1989).

Compounds from higher plants are of great potential value as medicines, as well as starting points for synthetic analogues and industrial raw materials. In contrast to synthetic drugs, medicinal plants play an important role for the basic health care level of various developing countries. Also the WHO encourages all countries to preserve and use the safe and positive elements of traditional medicine in their national health systems (Akerele, 1992).

Previous researchers have reported antimicrobial activity of extracts of *P. sidoides* and *P. reniforme* and their constituents against few bacteria and fungal species. Kayser and Kolodziej (1995), isolated highly oxygenated coumarins from *P. sidoides* and
investigated the antibacterial activity of extracts and constituents of *P. sidoides* and *P. reniforme*. Latté *et al.*, (2000), isolated flavonoids and tannins from *P. reniforme* and also the unusual coumarin patterns of *P. sidoides* and *P. reniforme* were analysed and compared for therapeutic equivalence. The aim of the study was to detect the antibacterial, antifungal and antituberculosis activities of the plant species that are used in preparation of the herbal remedy ‘umckaloabo’ and also to isolate compounds present from the active extract and to subject the isolated compound to biological screening.

In this study, we investigated the selected plants for their antimicrobial assays against the bacteria responsible for bronchitis, fungal pathogens infecting the respiratory tract and also for tuberculosis. The plants selected showed antibacterial, antifungal and antituberculosis activity *in vitro*. Acetone and ethanol extracts of *P. sidoides* and *P. reniforme* were investigated against the causative agents of bronchitis. Only ethanol extract of *P. sidoides* and its combination with *P. reniforme* showed activity. For antifungal activity against fungal pathogens of the respiratory tract were inhibited by the acetone and ethanol extracts of *P. reniforme* and ethanol extract of *P. sidoides*. Antituberculosis assay of the two species showed that the acetone, chloroform and ethanol root and shoot extracts of *P. reniforme* and its combination with *P. sidoides* inhibited *M. tuberculosis*. Our results do confirm the findings of other researchers who evaluated the antibacterial properties of root extracts of these two plants. **However, this is the first report on the investigation of root extracts of these two plants against *M. catarrhalis*, *A. niger*, *F. oxysporum*, *R. stolonifer* and *M. tuberculosis**.

The coumarins and flavonoids isolated from the roots of *P. sidoides* did not show inhibitory activity against *M. tuberculosis*. As Mycobacteria are intracellular pathogens, antimycobacterial activities reported by anecdotal evidences may be due to either direct or indirect effects. In recent studies, chemical constituents and pharmacological studies have demonstrated antibacterial and immunomodulatory activities *in vitro* (Kolodziej *et al.*, 2003). Though the compounds in our study did not show antituberculosis activity, it
can be speculated that the anecdotal evidence of tuberculosis-patients could be due to an immunostimulant. The isolated compounds from *P. sidoides* have not been investigated as yet for intracellular TB activity. It is therefore, recommended that these compounds should be analysed for intracellular activity against *M. tuberculosis* in mice and / or human macrophages.
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APPENDICES - Publications

12.1 Publications resulting from this thesis:


12.2 Article in preparation:

S.P.N. Mativandlela, N. Lall, A.A.H. Huisen and J.J.M. Meyer. Antituberculosis activity of compounds isolated from Pelargonium sidoides DC.
Antibacterial, antifungal and antituberculosis activity of the roots of *Pelargonium reniforme* Curtis and *Pelargonium sidoides* DC.*

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**Abstract**

Root extracts of *Pelargonium reniforme* Curtis and *Pelargonium sidoides* DC. were evaluated against bacteria which cause bronchitis, three fungal pathogens and *Mycobacterium tuberculosis* using the agar diffusion and BACTEC radiometric method, at concentrations ranging from 5.0 to 0.5 mg/ml. The ethanol extract of the roots of *P. sidoides* inhibited the growth of *Haemophilus influenza*, *Moraxella catarrhalis* and *Staphylococcus pneumonia* at a concentration of 5.0 mg/ml. Both acetone and ethanol extracts of *P. reniforme* and only the ethanol extract of *P. sidoides* inhibited the growth of *Aspergillus niger* and *Fusarium oxysporum* significantly at a concentration of 0.5 mg/ml. Growth of *Rhizopus stolonifer* was suppressed by the ethanol extract of *P. reniforme* and *P. sidoides* at 5.0 and 1.0 mg/ml, respectively. Acetone, chloroform and ethanol extracts of *P. reniforme* showed activity against *M. tuberculosis* exhibiting a minimum inhibitory concentration of 5.0 mg/ml.

**Keywords**: *Pelargonium reniforme*; *P. sidoides*; Antibacterial; Antifungal; Antituberculosis

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*Submitted to South African Journal of Botany*
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Introduction

The importance of Pelargonium species (Geraniaceae) is well documented (Watt and Breyer-Brandwijk 1962, Hutchings 1996). The genus Pelargonium comprises of more than 250 natural species of perennial small shrubs, which are limited in their geographical distribution. About 80% of Pelargonium species are confined to the southern parts of Africa, while others occur in Australia, New Zealand and the far East. These species usually grow in short grassland and sometimes with shrubs and trees on stony soil varying from sand to clay-loam, shale or basalt. The plants are evergreen when cultivated, but die back in nature during droughts and winter (May to August) (Van der Walt and Vorster 1985).

P. reniforme Curtis and P. sidoides DC. are highly valued by traditional healers for their curative properties and they are well known to generations of Khoi / San and Xhosa (South African tribes) traditional healers (Wagner and Bladt 1975). The Xhosa and the Zulu tribes of South Africa use these species to treat coughs, diarrhoea and tuberculosis (Watt and Breyer-Brandwijk 1962). The medicinally active ingredients are found in the bitter tasting roots of the plants (Helmstader 1996). A commonly used medicine produced in Germany, named, ‘Umckaloabo’ originates from the roots of P. sidoides and P. reniforme (Helmstader 1996, Kayser and Kolodziej 1998). This herbal medicine is extensively used in Germany for bronchitis, antibacterial and antifungal infections. Although this herbal medicine (Umckaloabo) is successfully employed in
modern phytotherapy in Europe to cure infectious diseases of the respiratory tract, the scientific basis of its remedial effect is still unclear (Kayser and Kolodziej 1995).

Bacteria, which are associated with either primary or secondary infections of bronchitis, are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. *H. influenzae*, a Gram-negative bacterium, is an obligate human parasite that is passed from person to person by way of the respiratory route. *M. catarrhalis*, a Gram-negative bacterium, causes bronchitis and pneumonia in children and adults. *S. pneumoniae*, a Gram-positive bacterium, infects the upper respiratory tract and can cause pneumonia, also it can infect the lining of the brain-spinal cord (meningitis), bones (osteomyelitis), joints (arthritis), ears (otitis media) and sinuses (sinusitis and bronchitis), (Benjamin *et al*. 1991).

*Apergillus niger*, *Fusarium oxysporum* and *Rhizopus stolonifer* are some of the fungal pathogens that can affect the respiratory tract. *A. niger*, is a causative agent of pulmonary diseases such as aspergillosis, bronchial asthma, acute allergic alveolitis etc. The fungus colonizes old tuberculous or bronchiostatic cavities, in which it forms a large colony (aspergilloma); or it may actually invades the lung tissue to produce haemorrhagic and necrotozing pneumonia (MacSween and Whaley 1992). *F. oxysporum* is responsible for fusariosis, skin infection, respiratory tract infections (tuberculosis and bronchitis) and arthritis and produces a 76% mortality rate in hospitalised immunocompromised patients (Monier *et al*. 1994). *R. stolonifer* causes mucorosis disease and it has been reported that
exposure to large numbers of *Rhizopus* spores has reportedly caused respiratory complications (Alexopoulos et al. 1996). Previously, researchers have reported antimicrobial activity of extracts of *Pelargoniums* and their constituents against a few bacterial (*St. aureus, S. pneumoniae, E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa* and *H. influenzae*) and fungal (*M. canis, M. gypseum, A. fumigatus, M. racemosus, R. nigricans*) pathogens as well as opportunistic yeasts such as *C. albicans, C. glabrata, C. krusei* and *Cryptococcus* species (Latté and Kolodziej 2000). Plant extracts of *P. reniforme* and *P. sidoides* have not been tested against the fungal pathogens, *A. niger, F. oxysporum, R. stolonifer* and the Gram-negative bacteria *M. catarrhalis*, which are indirectly responsible for the secondary infections of bronchitis and tuberculosis. In the present study, we have investigated their antimicrobial activity against the bacteria and fungi mainly responsible for bronchitis. We have also confirmed the findings of other researchers on the antibacterial activity of these species against *S. pneumoniae* and *H. influenzae*.

Tuberculosis (TB) kills approximately 2 million people each year, the global epidemic is growing and becoming more problematic. The breakdown in health services, the spread of HIV / AIDS and the emergence of multidrug-resistant (MDR) TB are contributing to the worsening impact of this disease. It is estimated that between 2002 and 2020, approximately a billion people will be newly infected, more than 150 million people will get sick, and 36 million will die of TB. The current threat in TB treatment lies in the emergence of strains resistant to two of the best antituberculosis drugs, isoniazid...
(INH) and rifampicin (RIF). The current TB-treatment comprises of 3-4 drugs for a period of 6-9 months (Bloom 2002). Novel drugs are required which can shorten this long treatment period and target multidrug resistant strains of TB. However, nothing had been published on antituberculosis activity of these plants. This is the first report on antituberculosis activity of these plants.

Materials and Methods

Plant material

Roots of *P. reniforme* and *P. sidoides* were collected from Qwaqwa, a region in the Free State province of South Africa. Voucher specimens of *P. reniforme* (P 092558) and *P. sidoides* (P 092559) were deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa.

Preparation of extracts

Dried and powdered roots of *P. reniforme* and *P. sidoides* (300g each) were extracted with 1L of acetone, chloroform and ethanol separately. The extracts were filtered and concentrated with a rotary vacuum evaporator to dryness at reduced pressure. For antibacterial and antifungal assays, acetone and ethanol extracts were dissolved in acetone to a concentration of 50mg ml⁻¹ and 100mg ml⁻¹ respectively. For the
antituberculosis assay, all 3 extracts were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 500mg ml$^{-1}$.

**Micro-organisms**

**Bacteria**

The bacteria used in this investigation *H. influenzae, M. catarrhalis*, and *S. pneumoniae* were obtained from the Department of Pathology, University of Pretoria, South Africa and maintained on Colombian agar.

**Antibacterial assay**

For the antibacterial assay, the minimum inhibitory concentration (MIC) of the acetone and ethanol extracts were determined by incorporating various amounts (5.0, 1.0 and 0.5mg ml$^{-1}$) of the extracts into chocolate agar in sterile bottles and placed in a water bath to prevent solidification, then withdrawn into petri dishes and left to solidify for four hours. The bacterial colonies were transferred into the sterile screw capped round tubes to which 5ml of the diluting fluid (saline) was added for achieving McFarland no.1 turbidity standard. Each suspension was streaked on petri dishes containing the extracts and the chocolate agar. The plates (three replications) were incubated at 25 $^\circ$C for 24 hours and antimicrobial activity was evaluated thereafter. Streptomycin sulphate added to chocolate
agar to a final concentrations of 0.5, 0.01 and 0.05mg ml\(^{-1}\) served as positive controls and three petri dishes containing only 500 µl acetone mixed with chocolate agar served as negative controls.

**Fungi**

The fungal pathogens used in the study, *A. niger*, *F. oxysporum* and *R. stolonifer* were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa. Each fungus was maintained on Potato Dextrose Agar (PDA) at ±25 °C.

**Antifungal assay**

For the antifungal assay, the required amount of acetone and ethanol extracts were added to sterile PDA in 5ml petri dishes before congealing to yield final concentrations of 5.0, 1.0 and 0.5mg ml\(^{-1}\). PDA plates with acetone and fungi served as controls. Once the agar had solidified, a 5mm plug of a seven-day old fungal culture was placed in the centre of the petri dish containing the extract-amended and unamended PDA plates. The plates were sealed with parafilm and placed in a 25 °C incubator. Fungal growth was measured on two diametric lines after 3, 6 and 9 days of growth. Each treatment was replicated three times. The results of six-day growth was statistically analysed using analysis of variance (ANOVA) and comparison of means by Duncan’s Multiple Range Test. The
value of bars within each fungus are a mean of three replicates and are significantly different $P < 0.01$ (Figure 1).

*Mycobacterium tuberculosis*

A drug-susceptible strain of *M. tuberculosis*, H37Rv obtained from American Type, MD, USA Culture Collection (ATCC), 27294, was used to investigate the activity of the plant extracts.

**Antituberculosis assay**

The radiometric respiratory technique using the BACTEC system was used for susceptibility testing of *M. tuberculosis* as described previously (Lall *et al*. 2001, Lall *et al*. 2003). Solutions of all the extracts were prepared in DMSO to obtain a concentration of 500mg ml$^{-1}$ and stored at $4^\circ$C until used. Subsequent dilutions were made in DMSO and added to BACTEC 12B vials containing 4 ml of 7H12 medium broth to achieve the desired final concentrations of 5.0, 2.5, 1.0 and 0.5mg ml$^{-1}$ together with PANTA (Becton Dickinson & Company, Ferndale, South Africa), an antimicrobial supplement.

Control experiments showed that the final amount of DMSO (1%) in the medium had no effect on the growth of *M. tuberculosis*. Anti-TB drugs; streptomycin (4µg ml$^{-1}$), isoniazid (2µg ml$^{-1}$), rifampicin (0.2µg ml$^{-1}$) and ethambutanol (6µg ml$^{-1}$) (Sigma Chemical Co., South Africa), were also tested against the H37Rv strain of *M. tuberculosis* which served as positive drug controls. A homogenous culture (0.1ml) of all
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the strains of *M. tuberculosis*, yielding $1 \times 10^4$ to $1 \times 10^5$ colony-forming units per millilitre (CFU ml$^{-1}$), was inoculated in the vials containing the extracts as well as in the control vials (Heifets *et al.* 1985). Three extract-free vials were used as controls (medium + 1% DMSO): two vials (V1) were inoculated in the same way as the vials containing the extracts, and one (V2) was inoculated with a 1:100 dilution of the inoculum (1: 100 control) to produce an initial concentration representing 1% of the bacterial population ($1 \times 10^2$ to $1 \times 10^3$ CFU ml$^{-1}$). The MIC was defined as the lowest concentration of the extract that inhibited > 99% of the bacterial population.

When mycobacterium grows in 7H12 medium containing 14 C-labelled substrate (palmitic acid), they use the substrate and $^{14}$CO$_2$ is produced. The amount of $^{14}$CO$_2$ detected reflects the rate and amount of growth occurring in the sealed vial, and is expressed in terms of the growth index (GI), (Middlebrook *et al.* 1977). Inoculated bottles were incubated at 37°C and each bottle was assayed every day to measure GI, at about the same hour(s) until cumulative results were interpretable. The difference in the GI values of the last two days is designated as ΔGI. The GI readings of the vials containing the test extracts were compared with the control vials (V2). Readings were taken until the control vials, containing a 100 times lower dilution of the inoculum than the test vials, reached a GI of 30 or more. If the ΔGI values of the vials containing the test extracts were less than the control vials, the population was reported to be susceptible to the compound. Each test was replicated three times.
Whenever results suggested contamination (e.g., large, rapid increase in GI), bottles were inspected and the organisms were stained by Ziehl-Neelsen stain to determine whether the visible microbial growth was a mycobacterium (Kleeberg et al. 1980). With this stain, the bacilli appear as brilliantly stained red rods against a deep sky-blue background. Organisms often have a beaded appearance because of their polyphosphate content and unstained vacuoles (Joklik et al. 1968).

Since anecdotal evidence suggests the use of a combination of ethanol extracts of two *Pelargoniums*, we also included the combination of ethanol extracts of both the roots for antimicrobial assay.

**Results and Discussion**

It was found from the antibacterial assay that the ethanol and acetone extracts of *P. sidoides* and its combination with *P. reniforme* was active at 5.0mg ml\(^{-1}\) against *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*. Activity of streptomycin sulphate was observed on each bacteria at 0.5, 0.01 and 0.05mg ml\(^{-1}\). Kayser and Kolodziej (1997), found inhibition of *S. pneumoniae* and *H. influenzae* at concentrations of 7.5 and 5.0mg ml\(^{-1}\) respectively by acetone extracts of roots of *P. reniforme*. There have been few reports of these bacterial organisms being susceptible to other plant extracts. Christoph et al. 2001 found antibacterial activity of Australian tea tree oil from *Melaleuca alternifolia* (Cheel) and niaouli oil isolated from *M. quinquenervia* at 0.01 (%v v\(^{-1}\)) against *M. catarrhalis*. Crude acetone extracts of *P. reniforme* was not active against these bacteria.
at the highest concentration (5.0mg ml$^{-1}$) tested, similar to the findings of Magama et al. 2002, using *Euclea crispa* extremely. Essential oils of *P. graveolens* were found to be inactive against *Moraxella* sp. (Lis-Balchin et al. 1998b). Gram-negative bacteria have been found to be less susceptible to plant extracts in earlier studies done by other researchers (Kuhnt et al. 1994; Afolayan and Meyer 1995). Various other species of *Pelargoniums* such as *P. tomentosum*, *P. Odaratissium*, *P. denticulatum* and *P. filcifolium* have been found to possess good antibacterial activity against Gram-positive bacterial species such as *Staphylococcus aureus*, *Proteus vulgaris*, *Bacillus cereus*, and *S. epidermidis* (Lis-Balchin et al., 1998a).

The antifungal assay of the acetone and ethanol root extracts of *P. reniforme* and ethanol root extract of *P. sidoides*, showed activity against the fungal pathogens tested at a concentration of 5.0mg ml$^{-1}$ (Figure 1: a-b). In *in vitro* antifungal bioassays conducted earlier, Latté and Kolodziej (2000), found that the aqueous acetone extracts of the roots of *P. reniforme* were less potent exhibiting a MIC of 8.0mg ml$^{-1}$ against the filamentous fungi (*Aspergillus fumigatus*, *Rhizopus nigricans*, *Penicillium italicum*) mold fungi and opportunistic yeasts tested. Other plant extracts have been found to be antifungal against the fungi tested in this study. Chandrasekaran and Venkatesalu (2004), investigated the water and methanol extracts of *Syzygium jambolanum* for antifungal activity against *A. niger* and *R. stolonifer* and the highest zones of inhibition were recorded at 1.0 and 0.5mg ml$^{-1}$ respectively. Chamundeeswari et al. 2004 found an MIC of 2.5mg ml$^{-1}$ when ethanol root extract of *Trewia polycarpa* was tested against *A. niger*. 

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The antituberculosis assay of extracts was interpreted on day five or six when the control vials (V2) reached a GI value of 30 or more (Table 1). Acetone, chloroform and ethanol extracts from the roots of *P. reniforme* showed inhibitory activity at 5.0mg ml$^{-1}$ against the drug-sensitive strain of *M. tuberculosis* but the combination of root extracts from both *Pelargoniums* did not show any synergy. Activities of the standard antituberculosis drugs (streptomycin, ethambutol, rifampicin and isoniazid), used as positive controls, were much stronger than those of the extracts. Our results are in agreement with previous reports on antituberculosis activity of water extracts of *Thymus vulgaris*, *Nidorella anomala*, *Cryptocarya latifolia* and acetone extract of *Rapanea melanophloeos* where MIC’s were also found to be 5.0mg ml$^{-1}$ against *M. tuberculosis* (Lall and Meyer 1999). Extracts of *P. sidoides* were not active against *M. tuberculosis* similar to the results obtained by Fabry *et al*. 1998 where methanol extracts of *Entada abyssinica*, *Terminalia spinosa*, *Harrisonia abyssinica*, *Ximenia caffra*, *Azadirachta indica* and *Spilanthes mauritiana* were found to be inactive against *M. tuberculosis* at concentrations ranging from 2.0 – 0.5mg ml$^{-1}$.

The bacteria and fungi inhibited in this study have been associated with infections of the respiratory tract, which the local inhabitants of South Africa treat using roots of *P. sidoides* and *P. reniforme*. Also, the fact that the extracts inhibited *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*, which cause bronchitis, provides some scientific rationale for the use of the extracts for tuberculosis, bronchitis and chronic asthma.
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Acknowledgements

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References


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Appendices


Helmstadter A (1996) Umckaloabo- Late vindication of a secret remedy. Pharmaceutical Historian 26: 2 - 4


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Appendices


Captions for figures

**Figure 1:** Antifungal activity of:

(i) *P. reniforme* acetone and ethanol extract

(ii) *P. sidoides* acetone and ethanol extract

The value of bars within each fungus are a mean of three replicates and are significantly different P < 0.01. * AC = acetone; ET = ethanol
Table 1: Antituberculosis activity of the root extracts against the sensitive strain (H37Rv) of *Mycobacterium tuberculosis* as determined by the radiometric method

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Sensitive strain</th>
<th>MIC(^a) (mg ml(^{-1}))</th>
<th>ΔGI(^b) values (mg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pelargonium reniforme</em> (acetone)</td>
<td></td>
<td>5.0</td>
<td>1.5 ± 0.7 (S(^c))</td>
</tr>
<tr>
<td><em>P. reniforme</em> (chloroform)</td>
<td></td>
<td>5.0</td>
<td>0.5 ± 0.7 (S)</td>
</tr>
<tr>
<td><em>P. reniforme</em> (ethanol)</td>
<td></td>
<td>5.0</td>
<td>2.5 ± 0.7 (S)</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (acetone)</td>
<td></td>
<td>5.0</td>
<td>-1.0 ± 2.8 (S)</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (chloroform)</td>
<td></td>
<td>5.0</td>
<td>1.0 ± 0.0 (S)</td>
</tr>
<tr>
<td><em>P. reniforme</em> + <em>P. sidoides</em> (ethanol)</td>
<td></td>
<td>5.0</td>
<td>1.5 ± 0.7 (S)</td>
</tr>
<tr>
<td><em>P. sidoides</em> (acetone)</td>
<td>na(^d)</td>
<td></td>
<td>35.5 ± 6.3 (R(^e))</td>
</tr>
<tr>
<td><em>P. sidoides</em> (ethanol)</td>
<td>na</td>
<td></td>
<td>276.0 ± 9.89 (R)</td>
</tr>
<tr>
<td><em>P. sidoides</em> (chloroform)</td>
<td>na</td>
<td></td>
<td>18.5 ± 4.94 (R)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>0.006</td>
<td>0.33 ± 0.0 (S)</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.0002</td>
<td>0.0 ± 0.0 (S)</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.002</td>
<td>4.0 ± 0.0 (S)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Minimum inhibitory concentration.

\(^b\)ΔGI value (mean ± SD) of the control vial was 20 ± 1.4 for the sensitive strain.

\(^c\)Susceptible.

\(^d\)Not active at highest concentration tested.

\(^e\)Resistant at the highest concentration tested.