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).

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

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

2.3 *P. reniforme* and *P. sidoides*

(a) (b)

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

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).

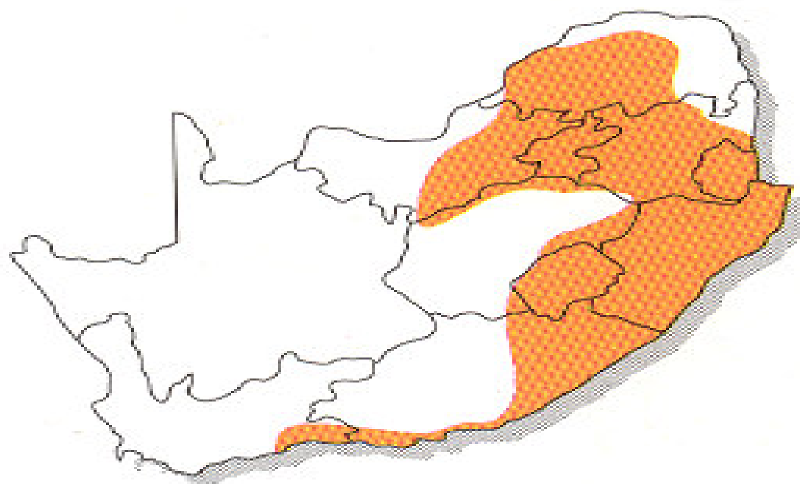

Figure 2.2. Distribution of *P. reniforme* and *P. sidoides* in South Africa

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).

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 distinct coumarins, such as umckalin, 7-acetoxy-5,6-dimethoxycoumarin, artelin**,** 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 strickly 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).

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 caryphyllene 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, astrigent, 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).

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 C_6-C_3-C_6. 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 antocyanins 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

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

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 complains, 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.

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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).

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

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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 ^{o}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

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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.

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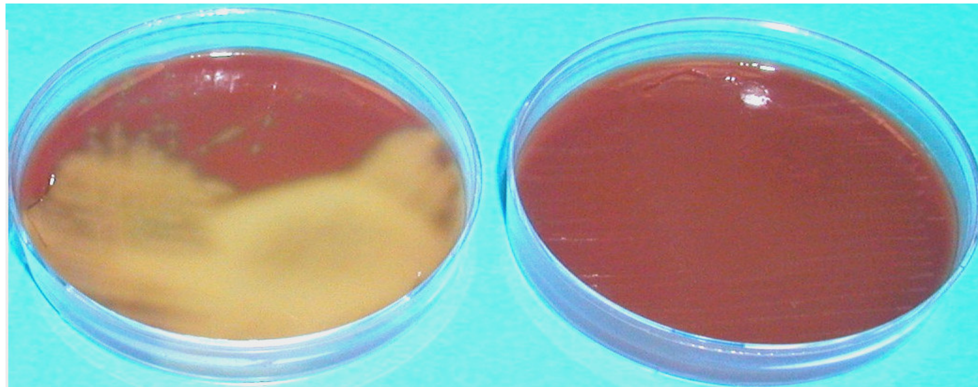

Control
H. influenza

Bacterial inhibition by acetone extract of
P. reniforme

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

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

Samples	MIC[a] (mg/ml) of samples against *H. influenza*, *M. catarrhalis* and *S. pneumonia*
***Pelargonium reniforme* (acetone)**	5.0 (N[b])
***P. reniforme* (ethanol)**	5.0 (N)
***P. sidoides* (acetone)**	5.0 (S[c])
***P. sidoides* (ethanol)**	5.0 (S)
***P. reniforme* + *P. sidoides* (acetone)**	5.0 (S)
***P. reniforme* + *P. sidoides* (ethanol)**	5.0 (S)
Streptomycin sulphate	0.01 (S)

[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-paranasal sinus mucosa. The warm, moist 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.

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The most commonly seen mycotic organisms in the Western world are *Alternarian*, *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 ^{o}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.

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

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

[a] Growth of fungal species after 72 hours (mean ± standard deviation).

[b] Acetone control.

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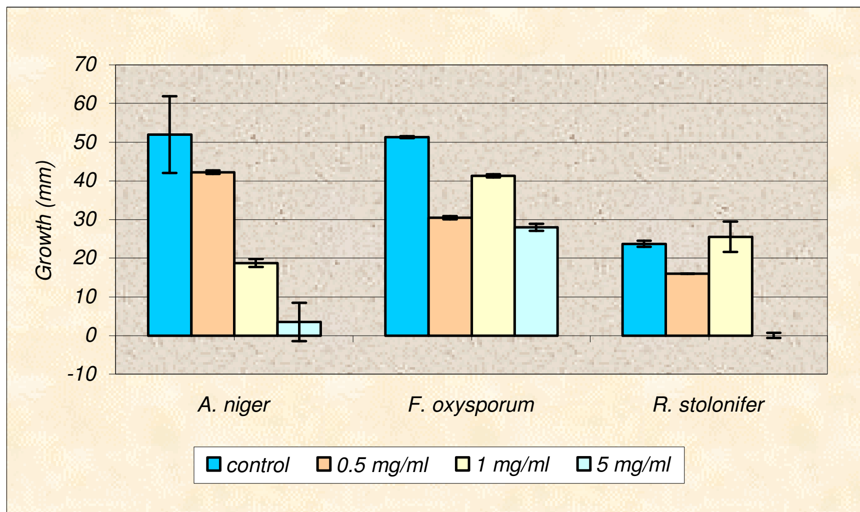
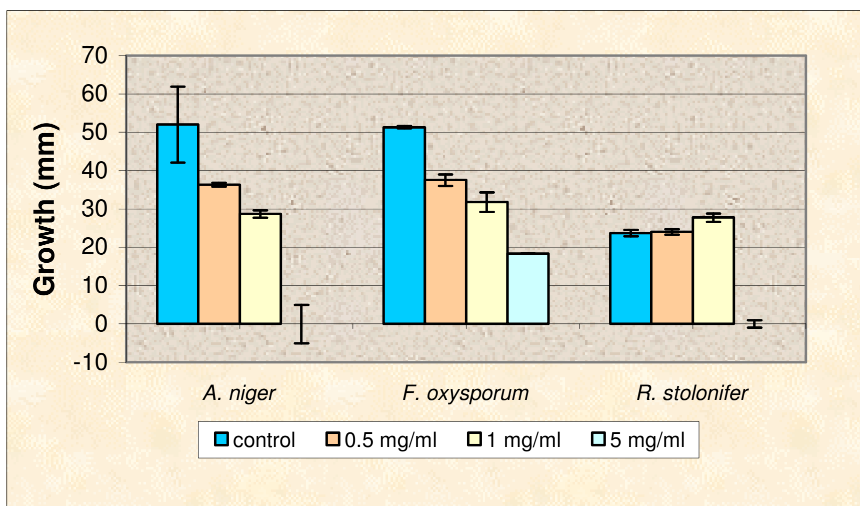
4.6 Fungal inhibition



(a)



(b)

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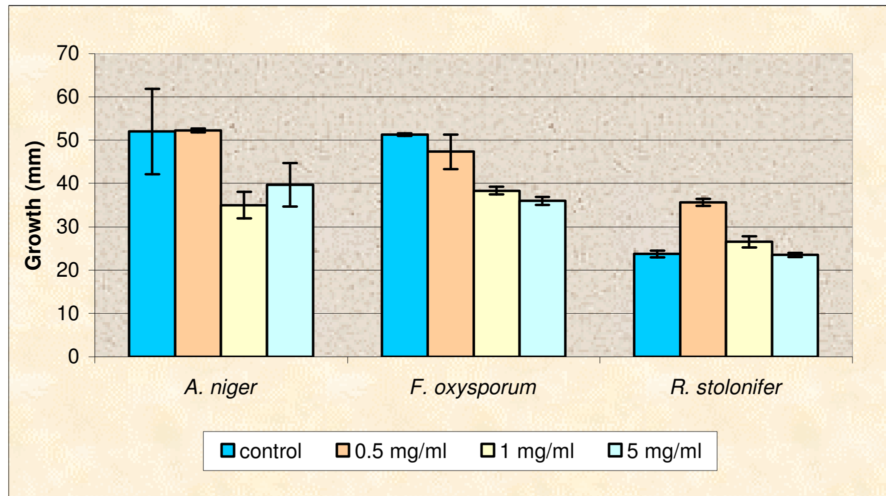
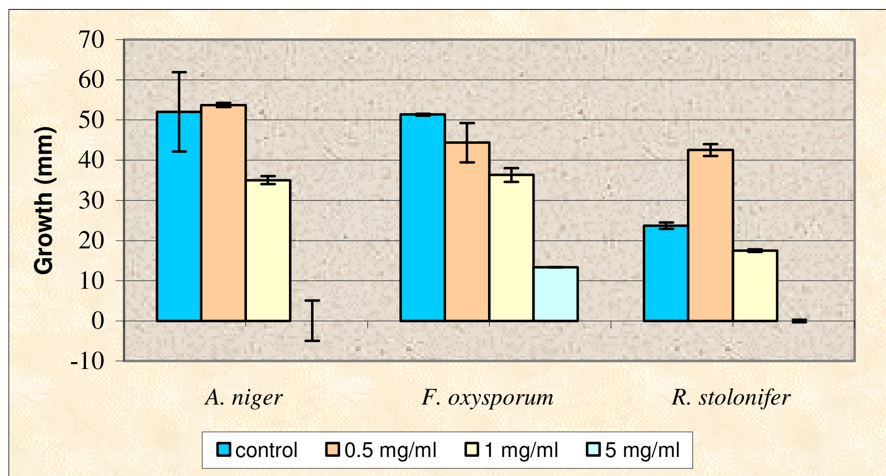


(c)



(d)

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

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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).

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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 sysytem 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

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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

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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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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.

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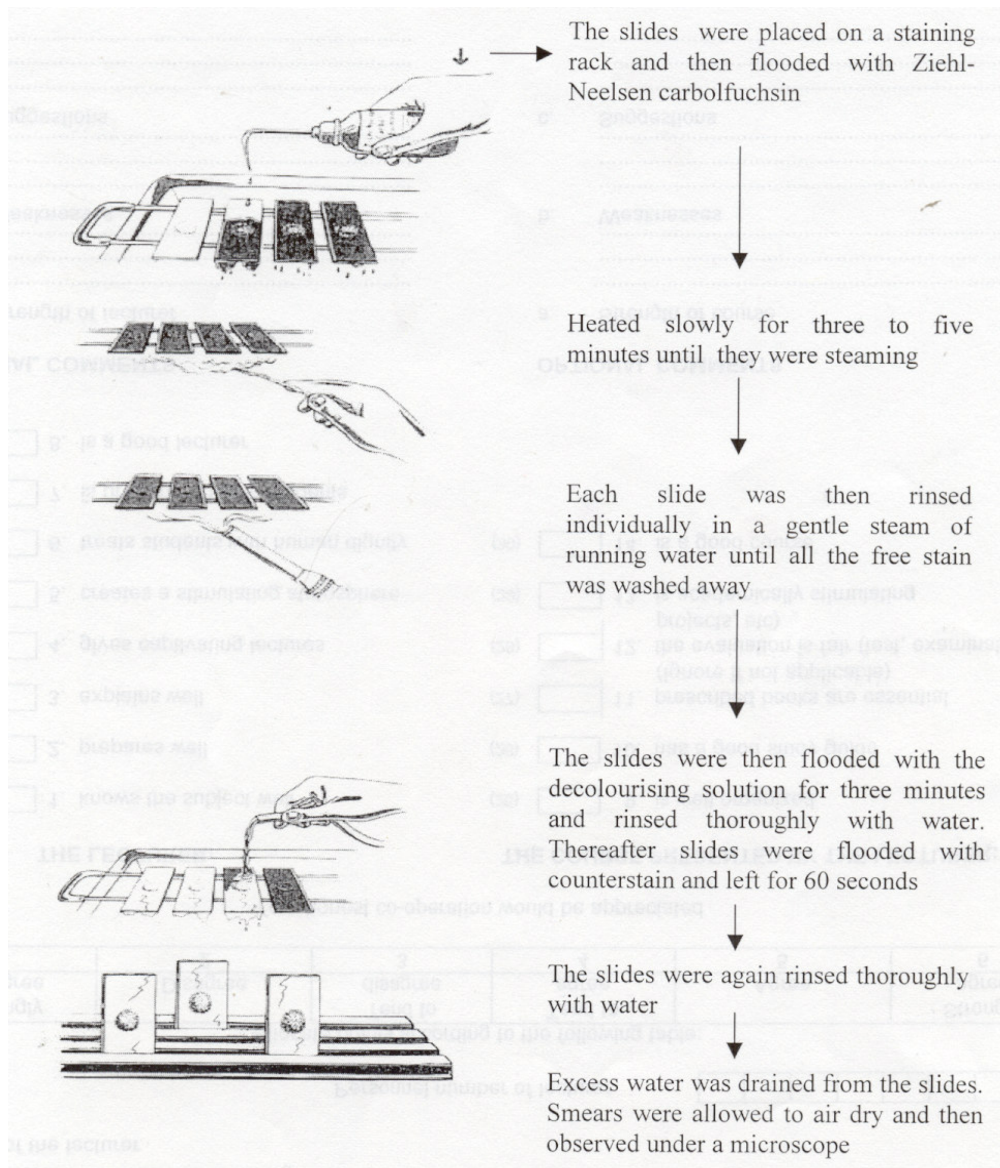

Figure 5.2 Ziehl-Neelsen staining (Kleeberg *et al*. 1980; WHO/ TB/97.258)

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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*.

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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

Samples	MIC[a] (mg/ml)	ΔGI[b]
Control		20 ± 1.4
***Pelargonium reniforme* (chloroform)**	5.0 (S[c])	0.0 ± 0.0
***P. reniforme* (chloroform)**	5.0 (S)	0.5 ± 0.7
***P. reniforme* (ethanol)**	5.0 (S)	0.0 ± 0.0
***P. reniforme* + *P. sidoides* (acetone)**	5.0 (S)	-1 ± 0.8
***P. reniforme* + *P. sidoides* (chloroform)**	5.0 (S)	1 ± 0.0
***P. reniforme* + *P. sidoides* (ethanol)**	5.0 (S)	1.5 ± 0.7
***P. sidoides* (acetone)**	5.0 (N[d])	35.5 ± 6.3
***P. sidoides* (chloroform)**	5.0 (S)	18.5 ± 4.94
***P. sidoides* (ethanol)**	5.0 (N)	276 ± 9.89
Streptomycin	0.004 (S)	5.0 ± 0.0
Ethambutol	0.006 (S)	0.33 ± 0.0
Rifampicin	0.0002 (S)	0.0 ± 0.0
Isoniazid	0.002 (S)	4.0 ± 0.0

[a] Minimum inhibitory concentration.

[b] Growth Index.

[c] Susceptible.

[d] Not active at highest concentration tested.

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

Samples	MIC[a] (mg/ml)	ΔGI[b]
Control		20 ± 1.4
***Pelargonium reniforme* (chloroform)**	5.0 (S[c])	1.5 ± 0.7
***P. reniforme* (chloroform)**	5.0 (S)	0.0 ± 0.0
***P. reniforme* (ethanol)**	5.0 (S)	0.0 ± 0.0
***P. reniforme* + *P. sidoides* (acetone)**	5.0 (S)	-1 ± 2.8
***P. reniforme* + *P. sidoides* (chloroform)**	5.0 (S)	1 ± 2.8
***P. reniforme* + *P. sidoides* (ethanol)**	5.0 (S)	2.5 ± 0.7
***P. sidoides* (acetone)**	5.0 (N[d])	28.0 ± 5.6
***P. sidoides* (chloroform)**	5.0 (N)	22.5 ± 12.0
***P. sidoides* (ethanol)**	5.0 (N)	36 ± 18.3
Streptomycin	0.004	5.0 ± 0.0
Ethambutol	0.006	0.33 ± 0.0
Rifampicin	0.0002	0.0 ± 0.0
Isoniazid	0.002	4.0 ± 0.0

[a] Minimum inhibitory concentration.

[b] Growth Index.

[c] Susceptible.

[d] Not active at highest concentration tested.

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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 intracellulary, 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).