CHAPTER 3
PHYTOCHEMISTRY AND MEDICINAL USES OF SELECTED MEDICINAL PLANTS

3.1. Introduction

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

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

3.2. Plants selected

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

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

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

Traditional healer questionnaire

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

2. Who collects the medicinal plants for you?

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

4. What plant parts do you use?

5. How do you package the plant materials?

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

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

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

9. Do you accept the substitutes as being effective?

10. Where do most of your patients come from?

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

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

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

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

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

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

3.2.1. *Artemisia afra* Jacq. ex Willd.

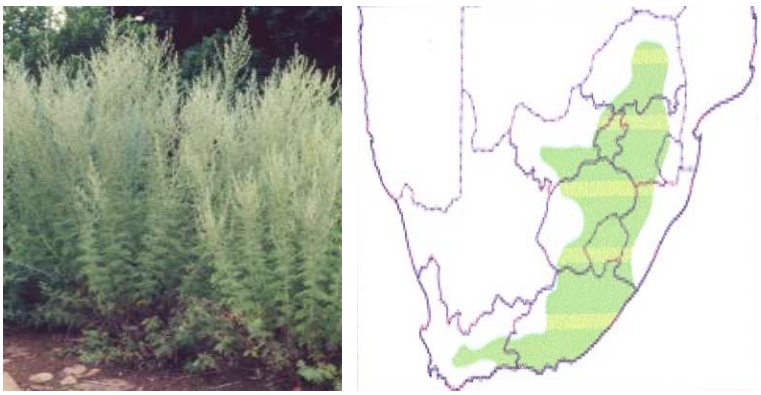

Figure 3.1. ***Artemisia afra*** **and distribution in South Africa (van Wyk *et al.*, 1997)**

3.2.1.1. Description and distribution

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

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

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

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

3.2.1.2. Medicinal uses

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

3.2.1.3. Phytochemistry and biological activity

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

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

3.2.2. *Dodonaea angustifolia* L. f.

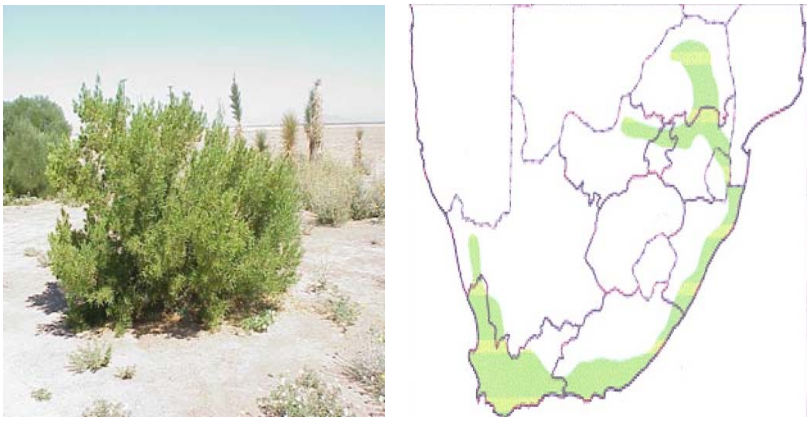

Figure 3.2. ***Dodonaea angustifolia*** **and distribution in South Africa (van Wyk *et al*., 1997)**

3.2.2.1. Description and distribution

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

3.2.2.2. Medicinal uses

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

3.2.2.3. Phytochemistry and biological activity

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

3.2.3. *Drosera capensis* L.

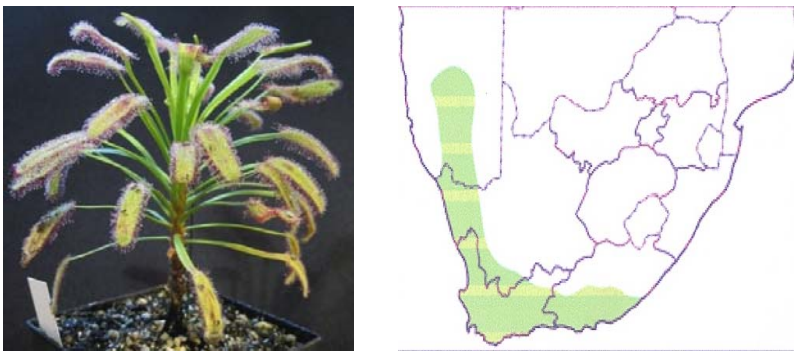

Figure 3.3. ***Drosera capensis*** **and distribution in South Africa (Gibson, 1993)**

3.2.3.1. Description and distribution

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

3.2.3.2. Medicinal uses

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

3.2.3.3. Phytochemistry and biological activity

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

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

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

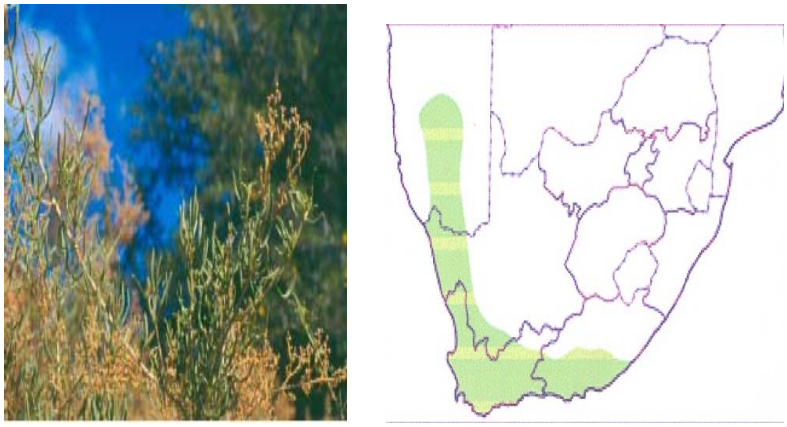

Figure 3.4. ***Galenia africana*** **and distribution in South Africa (van Wyk *et al*., 1997).**

3.2.4.1. Description and distribution

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

3.2.4.2. Medicinal uses

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

3.2.4.3. Phytochemistry and biological activity

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

3.2.5. *Prunus africana* Hook. f.

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

3.2.5.1. Description and distribution

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

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

3.2.5.2. Medicinal uses

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

3.2.5.3. Phytochemistry and biological activity

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

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

3.2.6. *Syzygium cordatum* Hochst. ex Krauss.

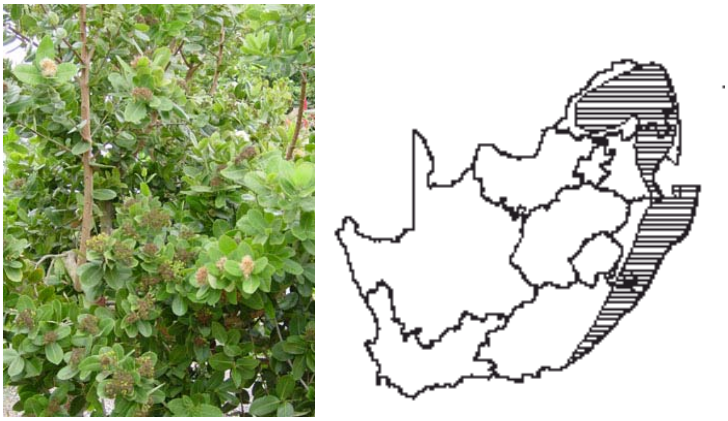

Figure 3.6. *Syzygium cordatum* and distribution in South Africa (van Wyk *et al*., 1997).

3.2.6.1. Description and distribution

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

3.2.6.2. Medicinal uses

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

*a*l., 2007).

3.2.6.3. Phytochemistry and biological activity

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

3.2.7. *Ziziphus mucronata* Willd.

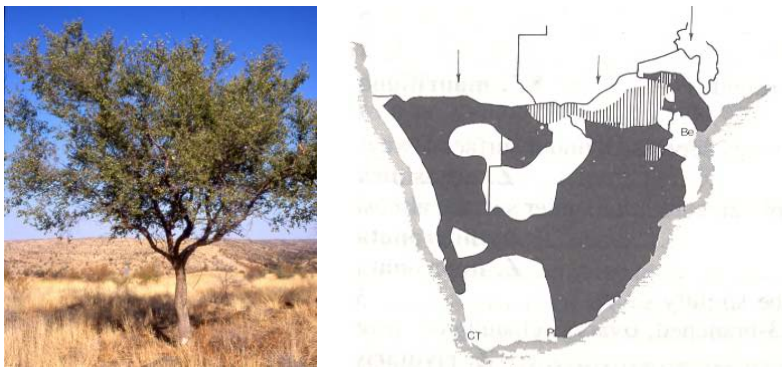

Figure 3.7. ***Ziziphus mucronata*** **and distribution in South Africa (van Wyk *et al*., 1997)**

3.2.7.1. Description and distribution

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

3.2.7.2. Medicinal uses

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

3.2.7.3. Phytochemistry and biological activity

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

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

3.3. Discussion and Conclusion

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

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

CHAPTER 4

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

4.1. Introduction

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

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

M. tuberculosis.

4.2. Materials and Methods

4.2.1. Plant materials

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

4.2.2. Preparation of plant extracts

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

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

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

4.2.3. Microorganisms

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

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

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

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

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

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

4.2.3.2. Microplate susceptibility testing against *M. smegmatis*

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

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

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

4.2.3.3. BACTEC: radiometric assay

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

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

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

Figure 4.1. BACTEC TB- 460 instrument

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

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

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

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

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

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

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

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

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

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

- **Ziehl-Neelsen carbolfuchin**

Fuchin

Basic fuchin 3.0 g

95% ethanol 100 ml

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

Phenol

Phenol crystals 5.0 g

Distilled water 100 ml

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

Working solution

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

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

Concentrated hydrochloric acid 3 ml

95% ethanol 97 ml

Concentrated hydrochloric acid was carefully added to 95% ethanol.

- **Counterstain: Methylene blue**

Methylene blue chloride 0.3 g

Distilled water 100 ml

Methylene blue chloride was dissolved in distilled water.

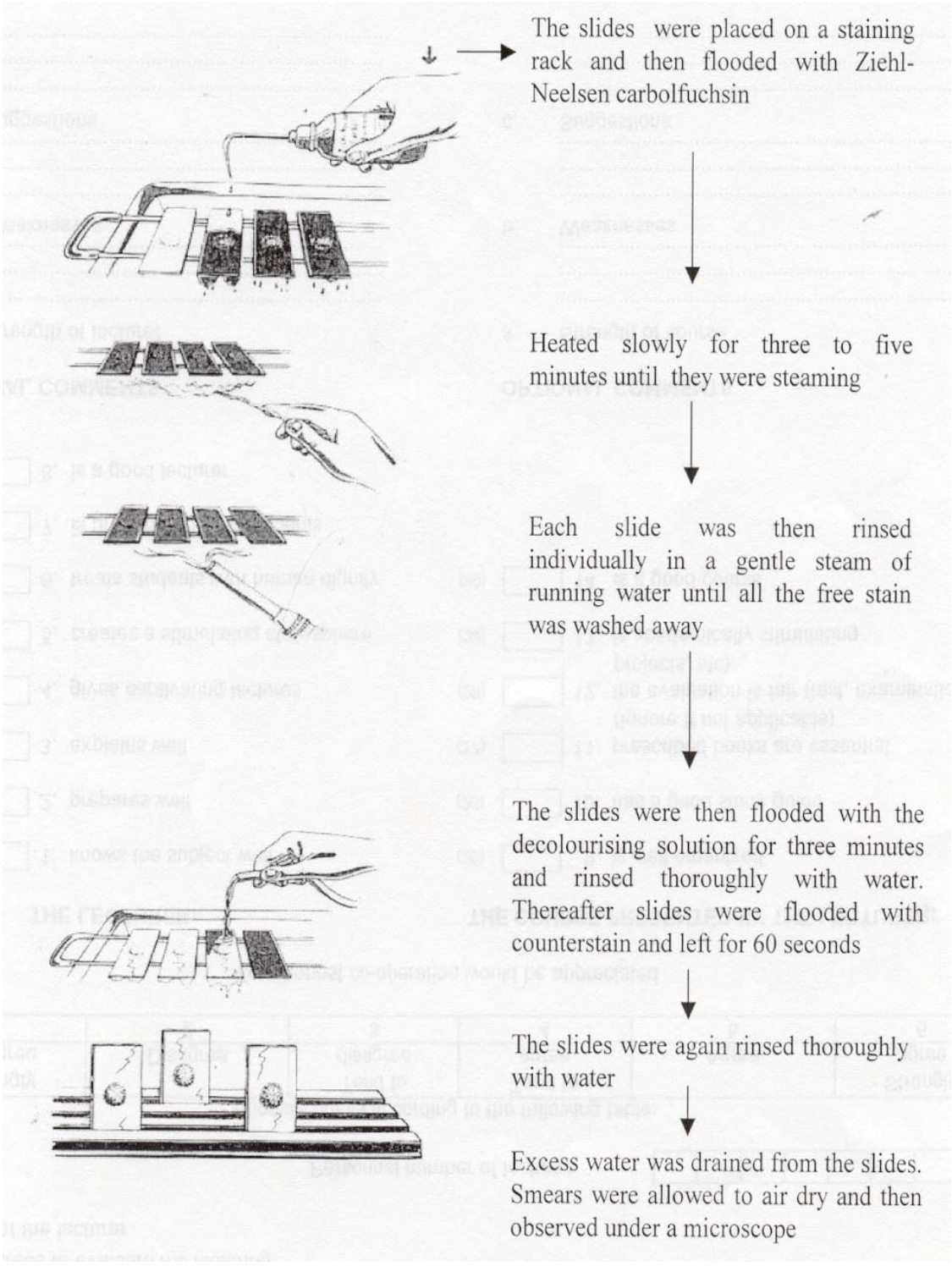

Figure 4.2. Ziehl-Neelsen staining (Kleeberg *et al*. 1980, WHO/ TB/97.258)

4.3. Results

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

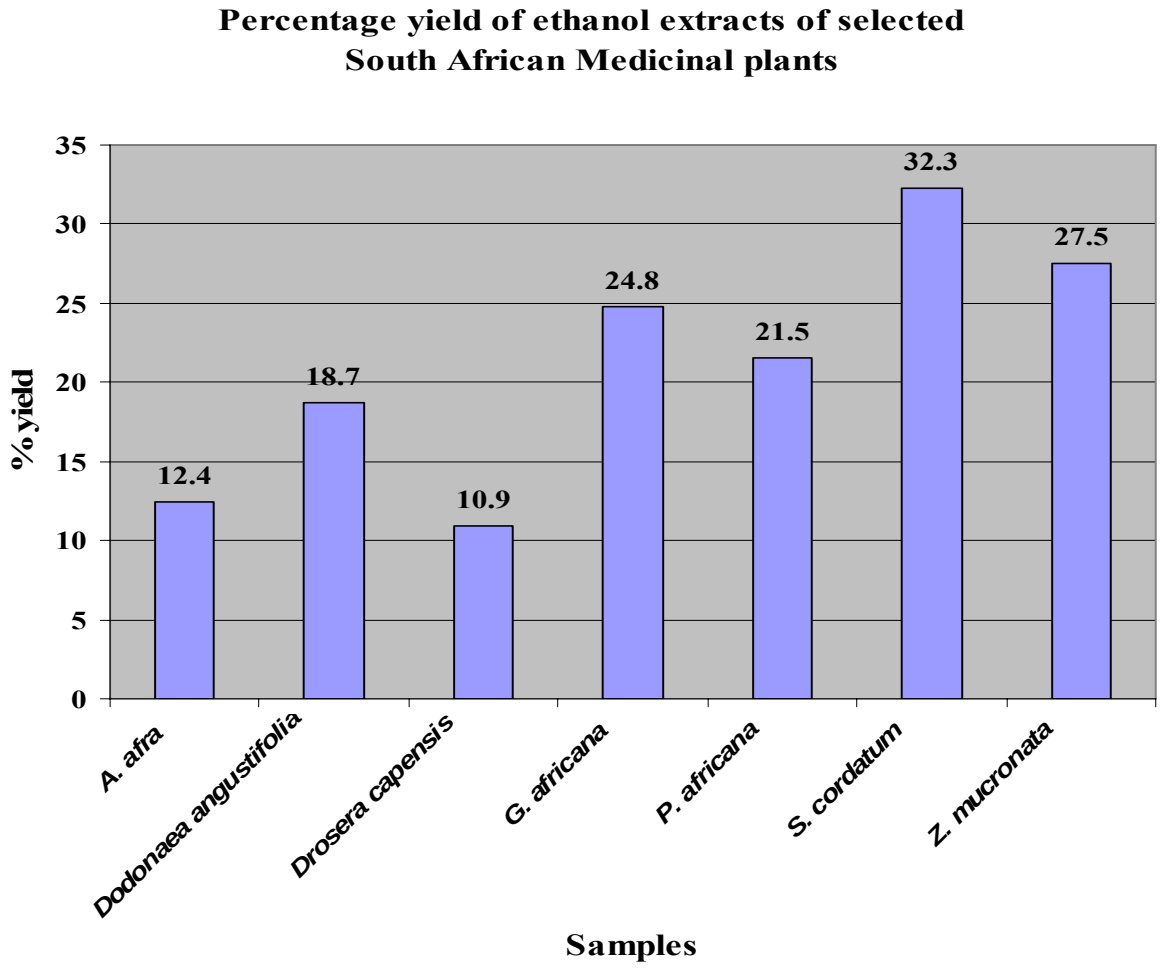

Figure 4.3. Percentage yield of ethanol crude plant extracts

4.3.1. Antituberculosis activity using the agar plate method

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

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

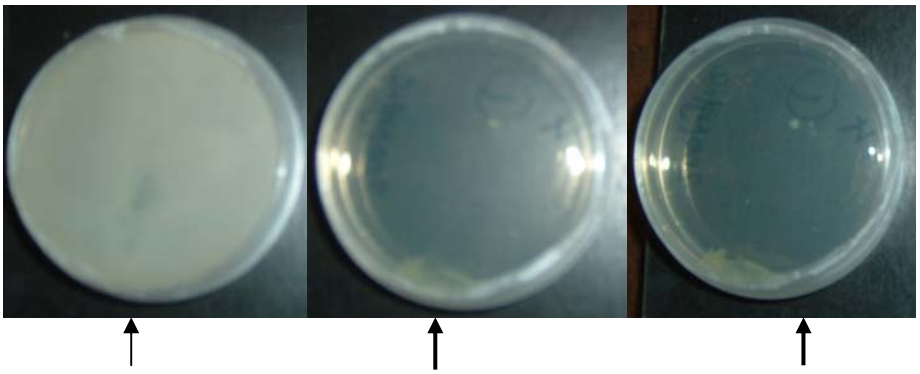

Figure 4.4. Antimycobacterial activity of ethanol extract of *G. africana* against *M. smegmatis* using the agar plate method at 50.0 mg/mL

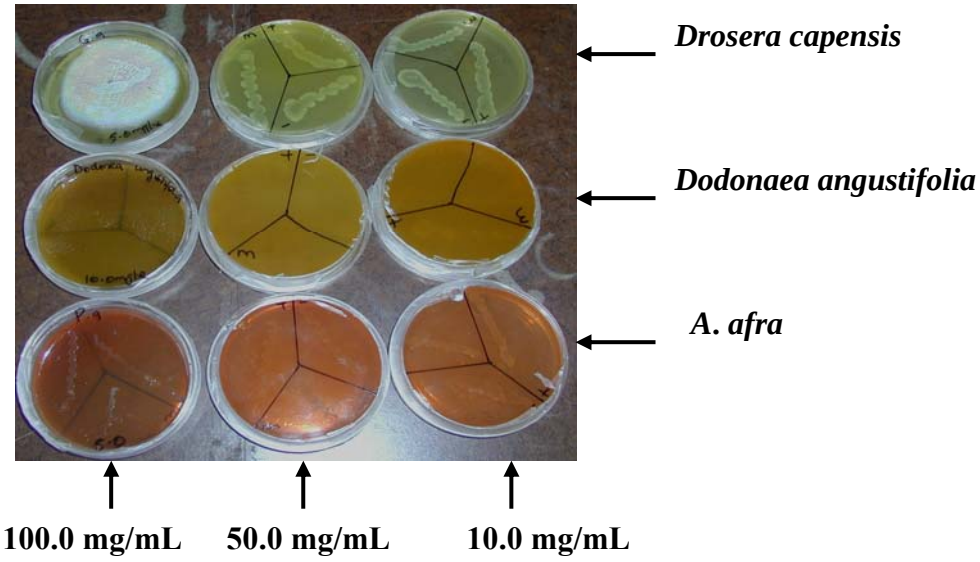

Figure 4.5. Antimycobacterial activity of *Drosera capensis*, *Dodonaea angustifolia* and *A. afra* against *M. smegmatis* using the agar plate method at 100.0 mg/mL

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

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

[a]Minimum Inhibitory Concentration.

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

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

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

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

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

[a]Minimum inhibitory concentration.

[b]Minimum bactericidal concentration.

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

[d]Not tested for MBC determination.

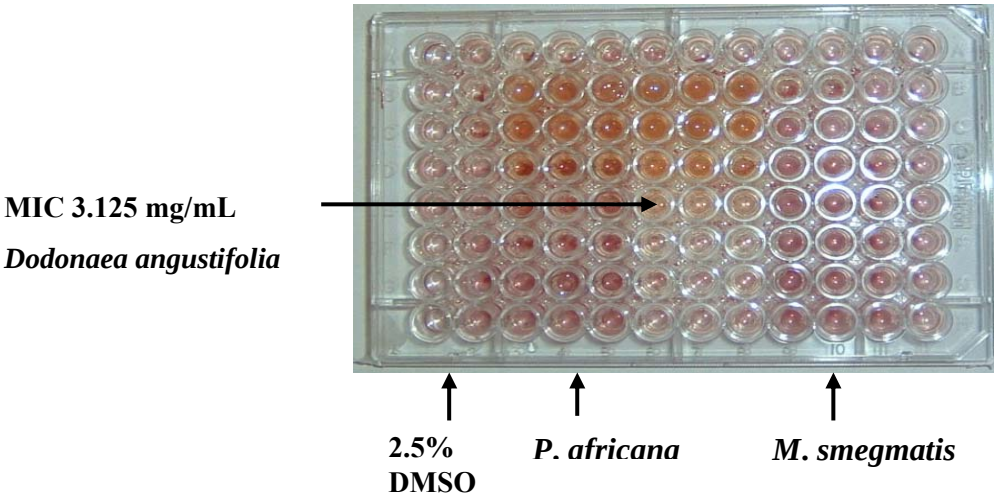

Figure 4.6. Microtitre plate exhibiting antimycobacterial activity against *M. smegmatis*

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

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

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

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

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

[a]Minimum inhibitory concentration.

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

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

[d]Susceptible.

4.4. Discussion and Conclusion

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

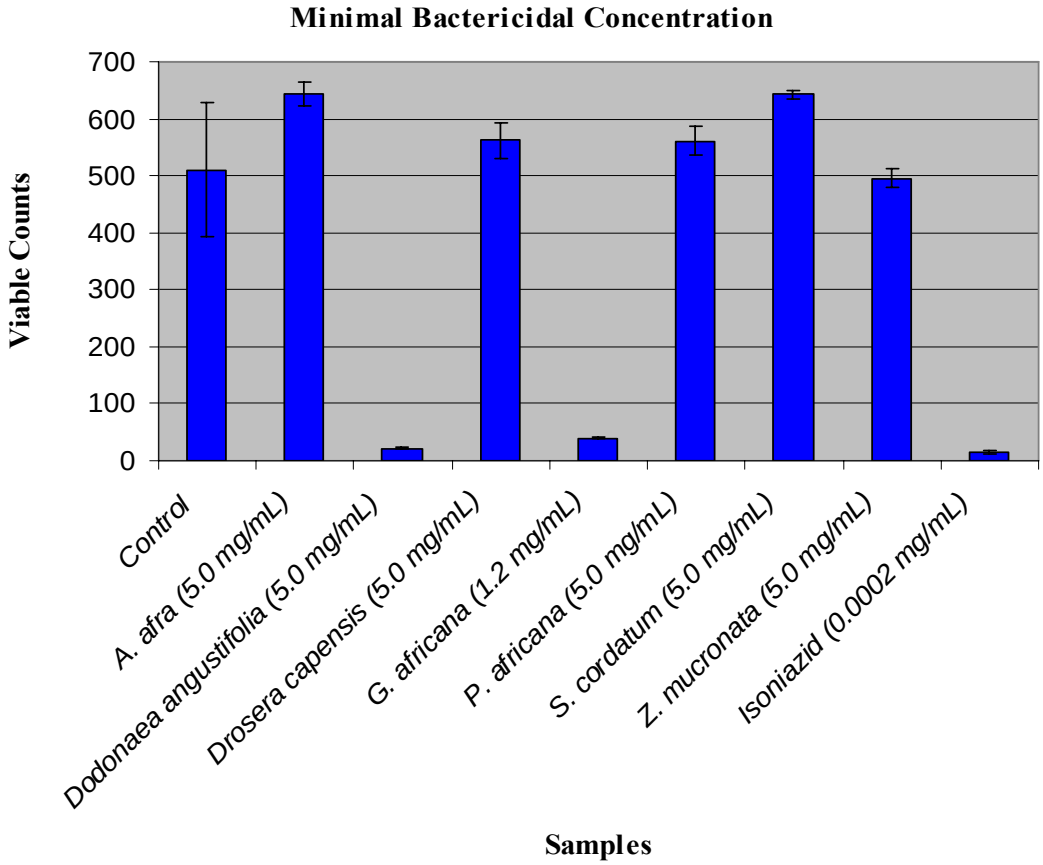

Figure 4.7. The comparative bactericidal effect of the active extracts against the drug-susceptibility strain of *M. tuberculosis*. Results illustrate the mean of viable bacterial counts ± standard error in the treated vials as compared to the untreated control vials

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

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

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

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

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

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

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