ANTIBACTERIAL ACTIVITY OF SELECTED PLANTS USED IN ETHNOVETERINARY MEDICINE

BY

SALOME MAMOKONE MAHLO

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCES, HEALTH AND AGRICULTURE
SCHOOL OF MOLECULAR AND LIFE SCIENCES
DEPARTMENT OF BIODIVERSITY
UNIVERSITY OF LIMPOPO

SUPERVISORS: MR PW MOKWALA
: PROF RV NIKOLOVA

April 2006

TABLE OF CONTENTS

i

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
EVM	ethnoveterinary medicine
MIC	minimum inhibitory concentration
PEG	polyethylene glycol
PVP	polyvinyl pyrrolidone
PVPP	polyvinyl polypyrrolidone
TLC	thin layer chromatography
WHO	world health organisation

ABSTRACT

ABSTRACT

The following plants namely, *Balanites maughamii*, *Breonadia salicina*, *Dombeya rotundifolia*, *Hyperacanthus amoenus* and *Piliostigma thonningii* were used for the determination of tannins and antibacterial activity using radial diffusion assay, agar-dilution and serial dilution methods, respectively. The investigation of these plant species is necessary for identifying effective plant species used in the treatment of various diseases of livestock in ethnoveterinary medicine. The antibacterial activity of different crude extracts of the five plant species has been successfully carried out using four bacterial strains (*Bacillus cereus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*). Acetone was used as an extraction solvent for preparation of all plant extracts for both tannin and antibacterial activity.

Air-dried plant materials (at room temperature, ± 25 ˚C) were used in the preparation of acetone extracts for all five plant samples. Comparative assessment of tannins in both 70% aqueous and 100% acetone extracts was used to select the most optimal extraction procedure suitable for both tannins and antibacterial assays. Plant extracted with 100% acetone showed highest levels of tannins as compared to 70% acetone.

The highest tannin levels in both 70% and 100% acetone extracts were recorded for *P. thonningii* while no tannins were detected in *B. maughamii*. Addition of PEG and PVPP to the extraction media resulted in the reduction of tannins in all crude plant extracts (in both 70% and 100%), with PEG being more effective than PVPP. One percent PEG removed the greatest quantity of tannins in all plant extracts, in comparison to 0.5% PEG (63.3%), and to 0.5 and 1% PVPP (24.8% and 49.4%, respectively).

The crude extracts from the leaves of *P. thonningii*, *D. rotundifolia* and *B. maughamii* and bark of *B. salicina* and *H. amoenus* were tested for *in vitro* antibacterial activity by agar-dilution and serial dilution methods. In serial dilution method, extracts of *B. maughamii* and *P. thonningii* showed the higher antibacterial activity (MIC 0.195 mg ml^{-1}) than *B. salicina*, *D. rotudifolia* and *H. amoenus* against *E. faecalis* and *P. aeruginosa*. In agar-dilution method, extract of *P. thonningii* showed the highest antibacterial activity

(MIC 0.5 mg ml^{-1}). Addition of 1% PEG to plant samples resulted in reduction of antibacterial activity in five plant extracts. The highest percentage reduction (97.8%) for *B. maughamii* against *E. faecalis* was obtained in the serial dilution method. The lowest percentage reduction was observed in agar-dilution against *E. faecalis* and *P. aeruginosa*.

A bioautographic assay showed the presence of compounds with antibacterial activity in all plant extracts and more antibacterial activity was observed in the ethyl acetate fraction of *B. maughamii*. The minimum inhibitory concentration (MIC) obtained in this study showed that plant extracts have antibacterial activity and support their use for the treatment of various diseases of livestock.

DECLARATION

I hereby declare that this dissertation submitted to the University of Limpopo for the degree of Master of Science in Botany is the result of my work and investigation, and that it has neither wholly nor partially been presented as a dissertation at this university or elsewhere.

Name: MAHLO SALOME MAMOKONE

Signature_____   Date:_____

DEDICATION

A special dedication to my parents (Wilson and Maria Mahlo) who stood by my side all the times through some difficult time and for encouraging me to achieve my degree and the love and support they showed me to reach this far in my studies.

ACKNOWLEDGEMENTS

I would like to acknowledge the following:

National Research Foundation (NRF) and Cannon Collins Educational Trust Fund of Southern Africa (CCETSA) for financial assistance.

Prof JW Ng'ambi from Department of Agriculture for the support you gave me through some difficult times, especially during the writing stages of my dissertation and for encouraging me to complete my Masters degree.

Small scale farmers from Basani village for providing information of plants used in Ethnoveterinary medicine, you are highly acknowledged.

My special thanks to Mr Sandrock, for his excellent contribution on taking and preparing pictures for my study.

To my parents (Wilson and Maria), sisters (Robina, Oinah, Hellen, Millicent and Mahlatse) and brothers (Godfrey, Jimmy, Peter, Frans and Prince) thank you for all your love, support and incessant prayers. I truly appreciate it.

Ms HK Mokoboki, from Department of Agriculture. Thank you for your excellent assistance with the statistical analysis and interpretation of data.

To a special friend Colleen, thank you for all your support and encouragement throughout the entire period of my studies, and thank you for believing in me!

Dr HR Chauke from Materials Modelling Centre, thanks for the support, advice and encouragement you always gave me throughout my project.

Lastly, I would like to thank the Almighty God for supporting, guiding and giving me strength to withstand the difficult moment and experiences throughout my studies. Thank you!

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

Ethnoveterinary medicine (EVM) refers to people's beliefs, knowledge, skills and practices related to the care of their animals (McCorkle 1986). An advantage of EVM is that they are locally available, easily accessible, and culturally appropriate and therefore readily understood and are also mostly given orally, and some are applied topically. However, there are some problems with EVM. These include lack of scientific validation of most of the ethnoveterinary therapies, seasonality of some medicinal plants, lack of integration with orthodox practises and difficulties in standardising herbal therapies as the concentration of an active ingredient varies in different parts of the plants (Swaleh 1999).

Written documentation of the uses and principle of medicinal plants is urgent because some plants are under threat of extinction. There is need to preserve and utilize indigenous medicinal knowledge (Ibrahim *et al.* 1984). In an attempt to conserve traditional knowledge, it is necessary that inventories of plants with therapeutic value are carried out, and the knowledge related to their use is documented in systematic studies. It is therefore important to conduct thorough investigations of as many traditionally used medicinal plants as possible before they are eradicated (McCorkle *et al.* 1996; Dold and Cocks 2001).

In South Africa, several species of medicinal plants are used by many ethnic groups for the treatment of various ailments in domestic animals. The treatment of livestock diseases using traditional remedies is widely practised in the rural communities (Masika *et al.* 2000). Some plant species are reported to possess diverse pharmacological properties. For example, they have antibacterial, antiviral, anthelmintic, immunomodulatory, antifungal, antidiabetic, acaricidal and insecticidal properties (Minja 2002). However, detailed information on such plants is missing.

2. Motivation

Many rural small-scale livestock farmers in South Africa still rely on ethnoveterinary medicine for the health of their livestock. Masika *et al.* (2000) found that 75% of rural livestock owners in the Eastern Cape province of South Africa use plants or plant based remedies to treat their livestock. It is, therefore, important that the use of medicinal plants should be validated and their efficacy proven or investigated so that effective health products can be made from some plants. It is also important to identify effective plant species among those that are used (CAF 2004). Such information can contribute to local empowerment and development of indigenous knowledge.

3. Aim

The aim of this study is to evaluate antibacterial potential of five medicinal plants used traditionally for the treatment of animal diseases.

4. Objectives

a. To determine the antibacterial activity of different crude extracts of selected plant species used in ethnoveterinary medicine in Limpopo province.

b. To fractionate crude plant extracts and determine the antibacterial activity of different fractions against selected bacterial strains using bioautography.

c. To determine the level of tannins and the effectiveness of precipitating agents, PEG and PVPP, on removal of tannins from different crude plant extracts in order to evaluate the effect of tannins on antibacterial activity.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Plants in traditional medicine

Traditional medicine is the total knowledge, skills and practices based on theories, beliefs, and indigenous cultural experience (whether explicable or not) used in the maintenance of health, diagnosing, preventing, or eliminating physical, mental or social diseases (WHO 2006). Such knowledge may rely exclusively on past experience and observations handed from generation to generation verbally or in writing. The prime advantage of traditional medicine is that it is an immediate, existing source of health care for people where they live. According to World Health Organization (WHO) more than 80% of the population in developing countries relies on plants as an integral part of their primary health care (Penso 1980). This also depends on whether the patients want to use biomedical or traditional health care (Gesler 1984, Dauskardt 1990).

Africa is a continent endowed with an enormous wealth of plant resources (Cunningham 1993) and South Africa, a country with a strong history of traditional healing, hosts a variety of around 30 000 plant species. In South Africa medicinal plants are an important part of the cultural heritage of the country and up to 60% of the population consult one of an estimated 200 000 traditional healers, in preference to or in addition to scientific medical doctors, especially in rural areas (Van Wyk *et al*. 1997). Although free health care has become entrenched in South Africa's constitution and many rural communities now have access to mobile clinics and hospitals, there is still, to a large extent, strong belief in herbal medicine, possibly due to an inherent distrust in anything "Western". Many rural people still rely on the cheaper traditional healing methods rather than the expensive treatments by scientific practitioners (Meyer and Afolayan 1995). Traditional plant uses can be linked directly to the utilisation and conservation of plants as well as the development of modern medicines (Masika *et al*. 2000).

Medicinal plants also play an important role in conventional scientific medicine. Scientific study of traditional medicines has focused on isolating bioactive components for possible use in modern drugs (Hoareau and DaSilva 1999) and there has seemingly been little effort to apply research results to the rational use of traditional medicines.

2.2 Ethnoveterinary Medicine (EVM)

The idea of using medicinal plants to treat livestock is not new (Cornell 2001). Since the domestication of livestock, herders and smallholders throughout the world have developed their own ways to treat their animals and protect them from the effects of diseases and weather (Von den Driesch 1989). These include plant-based remedies, surgical and manipulative techniques, husbandry strategies and associated magico-religious practises (McCorkle *et al.* 1996). Farmers obtain herbal remedies principally by preparing their own or by purchasing them from herbalists.

In South Africa, the treatment of livestock diseases using traditional remedies is widely ptactised in rural communities. In Limpopo province, Basani village, several species of medicinal plants have been reported to be used by small scale farmers for the treatment of various ailments in domestic animals (Personal communication).

Like scientific veterinary medicine, ethnoveterinary practices have been developed through trial and error. Ethnoveterinary medicine is developed by farmers in the fields and barns, rather than by scientists in laboratories and clinics. The need to validate ethnoveterinary medicine is important before they can be widely promoted (Mathias 2001). Livestock owners have an excellent knowledge of ethnobotany, which has formed the basis for screening plant materials as potential sources of medical drugs. Traditional healers have less to offer in the treatment and control of epidemic and endemic infectious diseases and they can cope with a reasonable spectrum of common diseases such as diarrhoea, wounds, colds, worms, coccidiosis, and reproductive disorders (Matekaire and Bwakura 2004). In recent years, increasing attention has been paid to ethnoveterinary

knowledge and local veterinary practices (Martin *et al*. 2001). There is a need to encourage resource poor farmers to use available resources and methods of veterinary medicine to improve productivity, but these, need to be assessed for effectiveness and safety.

2.3 Identification and treatment of livestock diseases

Disease concepts and treatments differ widely across societies, and even within a single community (Mathias 2004). The way that people in a community understand diseases determines the way that diseases are treated and the changes in the methods of treatment over time therefore suggest that disease perceptions are also dynamic (Van der Merwe *et al*. 2001). Many farmers treat their animals themselves before seeking help from outsiders, especially if they are familiar with the illness and can be diagnosed (Mesfin and Obsa 1994). Herdsmen and livestock owners normally can identify symptoms of diseases although some common infectious diseases have several signs and may affect different parts of the animal body. The symptoms for diseases that affect cattle are similar in different areas; however, the plant species used are usually different. This indicates that locally available plant populations are a major determinant of the plant species used in ethnoveterinary medicines. According to Marcus (1992) for some of the common diseases (anthrax, blackquaeters, bovine tuberculosis, contagious bovine pleuropneumonia and rinderpest), orthodox medicine is preferred while others are treated with combination of ethnoveterinary medicine and orthodox medicine. This is not surprising, especially in Africa, in the light of the cultural changes that are taking place (Prance 1994).

2.3.1 Plant uses in EVM

Plants comprise the largest component of the diverse therapeutic elements of traditional livestock health care practises. In South Africa, plant remedies are prepared in various ways including infusions, decoctions, ground fresh plant material or sap expressed from fresh material, charring and drying. Application of a remedy is by different routes and

methods, depending on the perceived cause of the disease and condition of the animal (Masika *et al*. 2000). Preparations are normally administered by adding different dried plant parts (bark, leaves, fruits and roots) to drinking water, but in some cases medicines are applied topically and this is the preferred form of administration for diseases of the skin. The most common form of plant remedy is a liquid for oral application. Liquid remedies for oral dosing are usually administered using a glass, cool-drink or beer bottle (Van der Merwe *et al*. 2001).

2.3.1.1 World wide

Medicinal plants are widely used in some countries as a primary source of prevention and control of livestock diseases. In Mexico, intestinal disorders in cows are treated with herbal extracts of *Polakowskia tacacco*. Ingredients from the mahogany tree (*Khaya senegalensis* A. Juss) are used to treat anthrax, diarrhoea, dysentery, footrot, helminthes infections, ringworm, to improve appetite and fertility, to relieve animals in cases of gastric, emetic and poisoning problems as well as a laxative (Hoareau and Dasilva 1999). In Zimbabwe *Aloe* species have been used to treat poultry diseases: *Aloe excelsa* is being used against fowl pox while *A. saponaria* is used to treat enteritis and indigestion (Bizima 1994). In Uganda, dietary supplements, such as vitamin A, in poultry feeds are supplied through *Amaranthus* supplementations (*Amaranthus spp)*. Coccidiosis and worm infestation in poultry is controlled with extracts from various plants that include *Khaya senegalensis, Solanum nodiflorum, Bozwellia dalzidi, Mimordia balasamia, Vitex doniana and Striga spp* (Atawodi *et al.* 2000).

In Kenya the Maasai people use a number of plants to treat livestock. For example roots of *Cissus quadrangularis* are boiled and given to cattle suffering from east coast fever. The bark and fruits of *Kigelia africana* are boiled and the extract used as remedy for gastroenteritis in calves. The bark of *Croton megalocarpus* is boiled and the resultant extract is given to livestock suffering from east coast fever or anthrax. The bark of *Tamarindus indica* is boiled and the resultant extract is used as a laxative for calves. The leaves of *Kedrostis foetidissima* are crushed and fed to cattle suffering from pasture bloat

and frothy bloat. The active ingredient is probably an antifoaming agent. *Albizia anthelmintica* is widely used in East Africa by poor smallholder farmers and pastoralists to treat their livestock against internal parasites (Bizima 1994).

2.3.1.2 South Africa

Different plant parts leaves, roots, bark, flowers, fruits and seeds and other above ground plant parts are reported to be used for various treatment of livestock in Eastern Cape, South Africa (Mirutse 2001). Dold and Cocks (2001) reported that the fresh leaves of *Aloe ferox* are put into poultry drinking water to prevent those contracting poultry disease and to prevent tick and lice infestation.

The use of sap from unripe *Solanum panduriforme* fruits is used to kill the microorganisms that cause diarrhoea in cattle (Dold and Cocks 2001). *Withania somnifera*, that is used to treat calf diarrhoea, is chemically complex. Its biological effects include: antibiotic, cytotoxic and anti-inflammatory activities. These effects could be used to rationalise its use in some types of diarrhea (Van Wyk *et al*. 1997; Bruneton 1999). A bark decoction of *Schotia latifolia* is used in the treatment of redwater in cattle. Plants which are used to treat retained placenta have a soapy sap like: *Dicerocaryum eriocarpum*, *Dicerocaryum senecioides*, *Pouzolzia mixta* and *Tribulus terrestris*, while *Aloe marlothii*'s leaf sap is slimy. Use of these plants may have its origin in the belief that plant characteristics can be transferred to patients. Previous reports also indicate the use of *D. eriocarpum* for retained placenta in cows and in women and as an aid to parturition in cows (Watt and Breyer-Brandwijk 1962; Mabogo 1990). *Sclerocarya birrea* and *Acacia karroo* are used to treat long bone fractures due to the bark's tough and fibrous structure (Van der Merwe *et al*. 2001).

2.3.1.3 Limpopo province

The following plants have been reported to be used as medicines for treatment of various diseases of livestock by small scale farmers at Basani, Limpopo province, South Africa (personal communication).These include: *Balanites maughamii*, *Breonadia salicina*, *Dombeya rotundifolia*, *Hyperacanthus amoenus* and *Piliostigma thonningii*.

Table 2.1 Selected plant species used in ethnoveterinary medicine in Limpopo province by small-scale farmers, their voucher specimen, different plant parts used, preparation and conditions.

Species	Voucher specimen	Plant part used	Preparation	Condition(s)
Balanites maughamii	Mahlo 1	Leaves	Leaf decoction	Diarrhoea in cattle
Breonadia salicina	Mahlo 2	Bark	Bark maceration	General intestinal diseases and retained placenta in cattle
Dombeya rotundifolia	Mahlo 3	Leaves	Leaf decoction	Diarrhoea in cattle
Hyperacanthus amoenus	Mahlo 4	Bark	Bark maceration	Relieving pain, loss of appetite and general ailments in cattle
Piliostigma thonningii	Mahlo 5	Leaves	Leaf decoction	Diarrhoea in cattle

2.3.1.3.1 Plant characteristics

(a) *Balanites maughamii*

i. Classification

B. maughamii belongs to the family Balanitaceae. This is a small family represented by four species of *Balanites* (*B. aegyptiaca*, *B. maughamii*, *B. pedicellaris* and *B. roxburghii*). The family is often included in the Zygophyllaceae (Van Wyk and Van Wyk 1997).

ii. Distribution

Balanitaceae family is Native to the Sudano-Sahelian zone, Israel and Jordan and is also found on black soils in Kenya. It is confined to the extreme eastern parts of South Africa, KwaZulu-Natal, Limpopo, Mpumalanga provinces and Mozambique (Von Breitenbach 1986). It is largely confined to the eastern sand, forests-tropical and dry forest. It occurs in dry bushveld and sand forests, often along riverbanks and it prefers clay loam soil.

iii. Description

The *Balanites* species are easily distinguished by their stalked, 2-foliate leaves. *Balanites maughamii* is a tall deciduous tree with the trunk distinctively and deeply fluted in old specimens (Figure 2.1). It has branchless grey-green, zigzag spines which are usually unequally forked. The leaflets are shortly stalked, grey-green, leathery and velvety hairy at least on the lower surface. Flowers occur in axillary clusters, are small and yellowish green. Petals are densely hairy on the outer surface. Fruit kernels yield a tasteless and odourless fixed oil (Watt and Breyer-Brandwijk 1962).

iv. Uses

Roots of *B. maughamii* are pounded and made into a medicine for apparently driving out demons (Liengme 1981). Zulu people use the bark to make an exhilarating bath. Another medicinal usage is that the bark decoctions are administered as emetics in unspecified parts of southern Africa.

Balanites maughamii tree is used locally for many products and these include, the wood is used for making tools and furniture, the fruit for sweets and alcoholic beverages, and the kernels for cooking oil and medicines. Thorns are traditionally used as protective charms for the homestead and fruit is used as an arrow poison by the Vhavenda (Mabogo 1990). The fruits of *Balanites* species are eaten by humans and animals but kill aquatic fauna and small fish. These species contain numerous steroidal glycosides derived from diosgenin and structurally related sapogenins. An example is cryptogenin. Activities that have been ascribed to saponins include anti-fungal, anti-viral, spermicidal and molluscicidal effects. The bark and fruit have mild molluscicidal properties whereas leaves and seeds have no molluscicidal properties (Pretorius *et al*. 1988).

(b) *Breonadia salicina*

i. Classification

Breonadia salicina belongs to the family Rubiaceae. This is the largest family of flowering plants in the order Gentianales. Six thousand species in 500 genera constitute one of the largest families of plants. Rubiaceae are mainly tropical woody plants and consist mostly of trees and shrubs, less often of perennial to annual herbs, as in Rubieae (subfamily Rubioideae) which are found in temperate region (Mongrand *et al*. 2005) and there are a few arctic species. Several are myrmecophilous (inhabited by ants). Three of the 22 Rubiaceae genera (Hydnophytum, Myrmecodia and Neonauclea) are known to have extensive radiations of myrmecophytic species. These Rubiaceae *myrmecophytic* species are presently classified in 11 different tribes and in all three subfamilies,

indicating multiple origins of myrmecophytism within the family (Razafimandimbison *et al*. 2005).

ii. Distribution

The Rubiaceae family is predominantly distributed in Southern Asia, with only a few species in mainland Africa and Northern Australia (Razafimandimbison *et al*. 2005). Exceptionally, few myrmecophytic rubiaceous species are known in the Neotropics despite its remarkably high species richness in Rubiaceae. In South Africa *B. salicina* is found on alluvia of rivers (from Limpopo River in the north to the Amatigulu River in Zululand in the South) and grows next to rivers and prefers sandy soils. The largest and best-developed patches are found in Maputaland (De Moor *et al*. 1977; Moll 1978; Moll and White 1978; Furness and Breen 1980).

iii. Description

Breonadia salicina is a small to very large (up to 40m) evergreen tree with a tall, straight trunk and rather narrow crown (Figure 2.2). Leaves are usually 4-whorled and clustered towards the ends of branches. They are hairless with thick petioles that are up to 20mm long. The flowers occur in compact, clusters fruits are very small capsules which are clustered in fruiting heads. It has seeds which are very small (Van Wyk and Van Wyk 1997).

iv. Uses

The leaves of *B. salicina* are browsed by game. The Zulu people use the bark for stomach complaints and the Vhavenda use root decoctions for the treatment of tachycardia (Arnold and Guluman 1984). The bark of *B. salicina* is reported to be astringent (Doke and Vilakazi 1972). The wood is pale to dark brown with pale flecks. It is durable and used for furniture, boats, floors and various types of construction work.

(c) *Dombeya rotundifolia*

i. Classification

Dombeya rotundifolia belongs to the family Sterculiaceae which occurs throughout the world (Palmer and Pitman 1961). This family is often referred to as the chestnut family. It comprises approximately 1 200 species (50 genera), mainly trees and shrubs found in tropical and subtropical regions (Reid *et al*.2001).

ii. Distribution

In South Africa, *D. rotundifolia* is found in woodlands over a wide range of altitudes in Kwazulu-Natal, Mpumalanga and Limpopo provinces. It is more abundant in the warmer, drier habitats (Immelman *et al*. 1973) and usually grows amongst rocks in sandy, gravelly brown soil.

iii. Description

Dombeya rotundifolia is commonly known as the wild pear, is a single–stemmed deciduous tree that grows to about 5-6m tall, with a moderate, irregular-shaped canopy (Immelman *et al*. 1973). Leaves are broadly ovate to almost circular, green and hairy (Figure 2.3). Flowers occur in axillary clusters before the appearance of the new leaves in early spring. Fruit is a capsule.

iv. Uses

The aqueous infusions of the bark or wood of *D. rotundifolia* are used as enemas or are taken orally for the treatment of intestinal ulcers, headaches, stomach complaints, haemorrhoids and diarrhoea (Watt and Breyer-Brandwijk 1962, Coates-Palgrave *et al*. 1985, Hutchings *et al*. 1996, Van Wyk *et al*. 1997, Thomas and Grant 1998). The bark is also used to treat heart problems and nausea in pregnant women. The roots are made into a tonic and administered as enemas for dyspepsia and sharp pains in the stomach. In Tanzania, the Zigula use the roots as a remedy for abdominal pains. It is also used as a

colic remedy. In Zambia, the leaves are rubbed on abscesses as a counter irritant (Watt and Breyer-Brandwijk 1962). This plant species serve as sources of medicines, fiber, firewood, timber for furniture, and as decorative plants. The wood of *D. rotundifolia* is heavy, tough, fine-textured, and used for implement handles and ornaments.

(d) *Hyperacanthus amoenus*

i. Classification

Hyperacanthus amoenus belongs to the family Rubiaceae (Van Wyk and Van Wyk 1997). Note that this plant species belong to the same family as *B*. *salicina* and share the same characteristics as discussed in detail above (classification of *B*. *salicina*).

ii. Distribution

Hyperacanthus amoenus is found in Mozambique, Swaziland and South Africa. In South Africa it occurs in Western Cape, KwaZulu-Natal and Limpopo provinces. The natural habitat of the shrub is mainly on mountain hill sides, where there is sandy or loam soil (personal communication).

iii. Description

Hyperacanthus amoenus is a shrub and it can be recognised by its opposite leaves and interpetiolar stipules (Figure 2.4). The leaves often have domatia in the axils of the side veins. Interpetiolar stipules occur between the opposite petiole bases, and often fall off at an early stage, leaving a distinct line or scar connecting the opposite petioles (Van Wyk and Van Wyk 1997).

iv. Uses

The root and fruit of *H. amoenus* are taken orally as emetics and for stomach complaints by the Zulu people (Pooley 1993). Fruit of *H. amoenus* are eaten by humans, monkeys and birds.

(e) *Piliostigma thonningii*

i. Classification

Piliostigma thonningii belongs to the family Caesalpiniaceae (Van Wyk and Van Wyk 1997). This is the largest family of trees in Southern Africa, comprising about 160 native species.

ii. Distribution

Piliostigma thonningii is widespread from Sudan southwards to Swaziland and Limpopo province of South Africa. These species grow in open woodland and wooded grassland of medium to low altitudes often on stream banks and prefer sandy soil.

iii. Description

This is a medium-size tree (Figure 2.5) and sexes are usually separate on different plants. The leaves are large (up to 120 mm long), thick and leathery. The venation is prominently raised below. Flowers occur in leaf axils. The leaf arrangement is opposed and the petals are white or pinkish. Pods are large, woody and indehiscent (Van Wyk and Van Wyk 1997).

iv. Uses

The bark, root or leaves are used in treating leprosy and small pox (Dalziel 1937). The root can also be used for treating coughs, while the powdered bark of the fresh inner bark of the pods are applied as a dressing for wounds and ulcers (Irvine 1961). A concentrated

infusion of the bark is used for inflamed gums as mouth wash. The cold infusion of the bark is used as an antidiarrhoeic and an antidysenteric medicine. The bark of *P. thonningii* contains 18-20% tannins and produces a red-brown dye and is also used in tanning leather. Farmers in the Limpopo province collect the pods of *P. thonningii* (which are rich in ascorbic acid) and grind them into a meal to feed their cattle during the dry winter months. The unripe pods can be used as substitute for soap.

Figure 2.1 *Balanites maughamii*

Figure 2.2 *Breonadia salicina*

Figure 2.3 *Dombeya rotundifolia*

Figure 2.4 *Hyperacanthus amoenus*

Figure 2.5 *Piliostigma thonningii*

2.4 Bioactive compounds from plants

Bioactive compounds found in plants are extremely important to human and animal health, and consequently to the pharmaceutical, food and feed industries. Some bioactive compounds are species-specific and others may be found in several or many plant species of a genus, in several related genera, or even families. These active constituents may be present in small quantities in plant species from which they were first discovered (Kris-Etherton *et al*. 2002). Other species in related genera may be superior sources of these compounds, or may lead to new bioactive derivatives (McGaw *et al*. 2002). When one considers that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries become evident (Guri-Fakim and Subratty 1996). The crucial factor for the ultimate success of an investigation into bioactive plant constituents is, thus, the selection of plant material. Many plants concentrate certain secondary metabolites in specific organs and variation in bioactivity is often encountered between different parts of the same plant (O'Neill and Lewis 1993). It is believed that bioactive compounds have an important ecological role. They can work as pollinator attractants and as chemical defenses against insects, herbivores and microorganisms.

Most bioactive/secondary metabolites are obtained directly from plant tissue (Ibrahim 2006). Secondary metabolites are substances that do not appear to have primary metabolic functions (photosynthesis, respiration, and carbon fixation) and which vary in their distribution in the plant kingdom (Harborne and Baxter 1999). Generally there are three major groups of secondary metabolites in plants including nitrogen containing compounds, terpenes, and phenolics.

2.4.1 Nitrogen containing compound

2.4.1.1 Alkaloids

Alkaloids are nitrogenous compounds and are well known for potent pharmacological activities (such as analgesics, anti-malarial, anti-spasmotics) and treatment of hypertension, mental disorders and tumours (Rajnikant *et al*. 2005). The availability of

nitrogen (N) is expected to play an important role in the biosynthesis and accumulation of alkaloids in plants. Nitrogen has been found to increase the content of alkaloids in some of the medicinal as well as non-medicinal plants such as tobacco, lupines, barley, *Datura, Atropa* and *Papaver*. The presence of alkaloids in some plant species can often indicate toxicity. The alkaloids appear to assist in the biosynthesis of some other compounds that are required for repair and cell-to-cell communication (Ghosal *et al*. 1990). They can act on the central nervous system. Their resemblance to the morphine and codeine skeletons, e.g. caranine, crinine, galanthamine may account for their analgesic activity (Ghosal *et al*. 1985). The majority of compounds found in the Amaryllidaceae family are alkaloids of the norbelladine type (Waller and Nowacki 1978). *Crinum* bulbs contain the highest concentrations of alkaloids. *Crinum* alkaloids play a role in the protective and repair mechanisms of the plant (Fennell and Van Staden 2001). Previous studies indicated that Amaryllidaceae alkaloids have antitumor potential and, amongst other characteristics, showed *in vivo* activity against various human viruses (Duri *et al*. 1994; Hutchings *et al*. 1996). The bulbs also contain flavonols, organic acids, carbohydrates and soluble nitrogen compounds (Waller and Nowacki 1978).

Periwinkle (*Catharanthus roseus)*, a medicinal plant native to Madagascar, is now found in many tropical and sub-tropical regions of the world. It derives its economic importance from its highly valued anti-cancer leaf alkaloids, vincristine and vinblastine and anti-hypertension root alkaloids and ajmalicine. The antimicrobial activity in species of the Piperaceae family has been found to be due to the amides, essential oil, lignans, phenylpropanoids, alkaloids, neolignans and chromenes (Masuda *et al*. 1991, Benevides *et al*. 1999, Dorman and Deans 2000).

2.4.1.2 Cyanogenic glycosides

Cyanogenic glycosides are widely distributed among families of flowering plants. They are present in seeds and have caused poisoning in herbivores (Henry *et al*. 1994; Gleadow and Woodrow 2002). Cyanogenic glycosides are protective compounds, not toxic, but are readily broken down to give off volatile poisons when the plant is crushed.

The biological function of cyanogenic glycosides has been reported to be difficult to assess (Jones 1998; Selmar 1999; Jones *et al.* 2000).

2.4.2 Terpenes

Terpenes or terpenoids are toxins and feeding deterrents to many plant-feeding insects and mammals (Taiz and Zeigher 2002). Many terpenes play important roles as plant hormones and in the chemical defenses of plants against microbial diseases and insect herbivores (Croteau 1998). Terpenes are used commercially as flavours and fragrances in foods and cosmetics. They are important for the quality of agricultural products, such as fruits and flowers. Terpenes are reported to have medicinal properties such as anti-carcinogenic, anti-malaria, anti-ulcer, hepaticidal, antimicrobial and diuretic activity (Aharoni *et al.* 2005). Leaves of members of the family Lamiaceae are known to contain terpenoids, which possess antifungal, antibacterial and anti-cancer activities (Cole 1992). Previous studies reported that *Micromeria graeca* have antibacterial activity due to monoterpenes in its essential oil (Marin *et al.* 2001). Leaves and flowers of *Ludwigia adscendens* contain terpenes and it has been previously reported that the leaves possess a strong antimicrobial activity (Ahmed *et al.* 2005).

2.4.3 Phenolic compounds

2.4.3.1 Flavonoids

Flavonoids are a group of low molecular weight chemical compounds, e.g., the phenylbenzopyrones, found in all vascular plants. They are common constituents of fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey (Grange and Davey 1990). The physiologically active constituents have been used to treat human diseases (Cushnie and Lamb 2005). They have been reported to possess many useful medicinal properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity (Havsteen 1983), antiallergic activity (Harborne and Baxter 1999), antioxidant activity (Middletone and Chitan 1993), vascular activity and cytotoxic antitumour activity (Harborne and Williams 2000).

Chemical investigations of *Helichrysum* species have revealed that they are rich sources of flavonoids that are probably used in chemical defense mechanisms of the plants against bacteria and fungi (Hilliard 1983). Flavonoids have been reported to occur in green teas and previous studies showed that teas exerted antimicrobial activity (Toda *et al*. 1989). Aerial parts of *Fraxinus ornus* contain flavonoids, and biological studies had revealed the antimicrobial, wound healing, anti-inflammatory, immunomodulatory and antiviral activities (Kostova 2001). The presence of flavonoids in the Punicaceae family can be responsible for their antimicrobial activity.

2.4.3.2 Lignins

Lignins are found in the cell walls of various types of supporting and conducting tissue, notably the tracheids and vessel elements of the xylem. Lignins are also a key component of water transport tissue and have protective functions in plants. Its physical toughness deters feeding by animals, and its chemical durability makes it relatively indigestible to herbivores (Taiz and Zeiger 2002). Lignins also play role in maintaining gastrointestinal function and health in humans (McDougall *et al*. 1996).

2.4.3.3 Tannins

Tannins are complex group of plant secondary metabolites, which are soluble in polar solutions and these are distinguished from other polyphenolic compounds by their ability to precipitate proteins (Silanikove *et al*. 2001). Tannins in plants occur widely in vascular plants, their occurrence in the angiosperms being particularly associated with woody tissues. They are found in approximately 80% of woody and 15% of herbaceous dicotyledonous species and can occur at high levels in some forages, feeds, foods and medicinal herbs (Bryant *et al.* 1992; Chung *et al.* 1998a). Examples of families of dicotyledons rich in tannins are: Leguminosae (*Acacia* species (wattle), *Sesbania* species; *Lotus* species (trefoil); *Onobrychis* species (sainfoin)), Anacardiaceae (*Scinopsis balansae* (quebracho)), Combretaceae (myrobalan), Rhizophoraceae (mangrove), and Myrtaceae (*Eucalyptus* species, *Mirtus* species (Myrtle) and Polinaceae (canaigre). The amount and type of tannins synthesized by plants varies considerably depending on plant

species, cultivars, tissues, stage of development, and environmental conditions (Cornell 2000).

Plant parts containing tannins include bark, wood, fruit, fruit pods, leaves, roots and plant galls. Tannins are present in the upper epidermis of the leaves. However, in evergreen plants, these secondary metabolites are evenly distributed in all leaf tissues. In the plant cell, tannins are located in the vacuoles, which keep them separately from the proteins and enzymes of the cytoplasm

The protein precipitation capacity of tannins has been suggested as an important factor in reducing the palatability of plants by herbivores (Robbins *et al*. 1987) and in protection of plants against predators. This capacity may vary depending on the chemical structure of the compound. When plant tissue is damaged during animal feeding, the tannin may react with the protein moiety of the cell enzymes (oxidoreductases) in the cytoplasm and in the cell wall making the protein less accessible to the digestive juices of the animal. By binding to cell walls, tannins also reduce the digestion of energy-rich products of microbial fermentation such as volatile fatty acids. This in turn may adversely affect the preference of the feed containing the tannins (Kumar and Vaithiyanathan 1990; Reed 1995)**.** Tannins may also bind to bacterial adhesions and so, interfering with the availability of receptors on the cell surface (Cowan 1999). Tannins at low concentrations may also reduce bacteriophages (bacterial viruses) which can cause a reduction in microbial efficiency through non-specific lysis of bacteria or have anti-protozoal activity (Makkar *et al*. 1995).

Plants containing more than 10% tannins may have potential adverse effects on humans including upset stomach, renal damage, hepatic necrosis, and an increased risk of esophageal and nasal cancer (Kemper 1999). Many human physiological activities, such as stimulation of phagocytic cells, host-mediator tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam 1996). In medicine, the tannin-containing plant extracts are used as astringents and diuretics (for example *Crataegus spp* and *Filipendula ulmaria*), anti-inflammatorys (*Camellia sinensis*), antiseptic (*Camellia*

28

sinensis), and haemostatic pharmaceuticals (*Polygonum aviculare*) against diarrhoea, nasopharyngeal tumors (*Acacia farnesiana*) (Haslam 1989; Saijo *et al*. 1989; Hatano *et al*. 1991; Okuda *et al*. 1991).

Chemically, there are two main types of tannins: condensed tannins (proanthocyanidins) and hydrolysable (gallotannins and ellagitannins) tannins. Condensed and hydrolysable tannins may occur in the same plant. Proanthocyanidins are more widely distributed than hydrolysable tannins.

i. Condensed tannins

Condensed tannins are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. The properties of condensed tannins depend on their structure in terms of monomer units (degree of hydroxylation and 2, 3-*cis*- or 2, 3-*trans*-stereochemistry), their degree of polymerization and the linkage-type between flavan-3-ols with a considerable range of structural variation (Meagher *et al*. 2004). The multiple phenolic hydroxyl groups of condensed tannins lead to the formation of complexes with proteins, metal ions and other macromolecules like polysaccharides. It is accepted that the interaction between proteins and condensed tannins is pH dependent. Each protein has a distinctive pH optimum (pH of 4.1 for bovine serum albumin (BSA) and pH 6.1 for fraction-1-leaf proteins). Condensed tannins can bind with protein at pH 3.5-7.5 to form condensed tannin-protein complexes, which dissociate and release protein at pH less than 3.5 (Jones and Mangan 1977). The reactivity of condensed tannins with molecules of biological significance has important nutritional and physiological consequences. Condensed tannins are found in gymnosperms and angiosperms and these secondary metabolites are also found in forage legumes, trees and shrubs (Bryant *et al*. 1992; Silanikove *et al*. 2001). Condensed tannins are used in medicine to aid the healing of wounds and burns. When applied to the skin, they produce an impervious layer under which the healing process can take place (Harborne and Baxter 1993). They are also thought to have some protective value against toxins when taken internally.

ii. Hydrolysable tannins

Hydrolysable tannins are molecules with a polyol (generally D-glucose) as a central core. They have a large number of free phenolic groups that form strong hydrogen bonds at multiple sites with proteins and carbohydrates (Haslam 1989). The hydroxyl groups of the carbohydrates are partially or totally esterified with phenolic groups like gallic acid or ellagic acid. These types of tannins are hydrolysed by mild acids or mild bases to yield carbohydrates and phenolic acids. They are also hydrolysed by hot water or enzymes (e.g. tannase). Hydrolysable tannins are present in low amounts in plants and are found only in dicotyledons (Haslam 1989 and Silanikove *et al*. 2001). They occur mainly in fruit or in forages. They are often present in leaves of trees and browse shrubs in tropical areas. Hydrolysable tannins are of pharmacological interest because of their antiviral and anti-tumor properties (Harborne and Baxter 1993). Hydrolysable tannins are toxic to ruminants. Microbial metabolism and gastric digestion convert hydrolysable tannins into absorbable low molecular weight metabolites. Some of these compounds are toxic (Makkar 2003).

2.4.4 Tannin-binding agents

Artificial polymers such as water-soluble polyvinyl pyrrolidone (PVP), water-insoluble polyvinyl polypyrrolidone (PVPP), and water-soluble polyethylene glycol (PEG) contain a large number of oxygen atoms capable of forming hydrogen bonds with the phenolic groups in tannins, and to precipitate them from solutions. This property of tannin-binding agents has been exploited for separation of plant metabolites and removal of tannins in tannin-rich environments (Jones 1965).

i. Polyvinyl polypyrrolidone (PVPP)

The binding of Polyvinyl polypyrrolidone (PVPP) to phenols takes place via hydrogen bonding which increases with the number of phenolic hydroxyl groups and is dependent on their positions (Andersen and Sowers 1968, Doner *et al*. 1993). In general tannins bind to PVPP to different extremes. Polyvinyl polypyrrolidone is reported to have low

binding affinity and to be less effective than PEG for binding tannins (Makkar *et al*. 1995).

ii. Polyethylene glycol (PEG)

Polyethylene glycol is a synthetic polymer for which tannins have a greater binding affinity than proteins (Makkar 2003). For most of the tannins, the increase of the molecular weight of the PEG also increases its binding capacity and it selectively binds with tannins. Previous studies showed that the binding efficiency of PEG is higher than that of PVPP and it should be preferred for complexing tannins (Makkar *et al*. 1995). Advantage of using PEG to remove tannins is that it forms tannin-protein complexes, as their affinity for tannins is higher than that for proteins. Polyethylene glycol is also used as additive to the drinking water of animals to bind tannins and reduce their anti-nutritional effect (Ford and Hewitt 1979; Nuñez-Hernández *et al*. 1991; McNabb *et al*. 1996; Yu *et al*. 1996; Barahona *et al*. 1997). Polyethylene glycol has been used in a variety of pharmaceutical preparations, and is relatively nontoxic (Wilson and Thomas 1984; Deng *et al*. 2005). It has been reported that PEG exerts antibacterial activity because it has a low water activity (Chirife *et al*. 1983).

2.5 Phytochemical analyses of bioactive compounds

2.5.1 Preparation of plant extracts for

2.5.1.1 Tannin assays

Generally, aqueous methanol (50%), aqueous acetone (70%) and acidic methanol are used as a solvent for extracting tannins from fresh or dry plant material (Hagerman 1988/ FAO/AEIA 2000; Cornell 2000).

2.5.1.2 Bioassays

Powdered dry or fresh plant materials are macerated or homogenized in organic or aqueous solvents and then filtered to prepare crude extracts for screening of antimicrobial activity. The choice of solvents depends on which antimicrobial components are screened

for. The effect of the extractant on subsequent separation procedure is not important, but the extractant should not inhibit the bioassay (Kotzé and Eloff 2002).

Cowan (1999) indicated that water, ethanol, methanol, chloroform, methylene dichloride and acetone have been used to extract and determine antibacterial activity from plants. Such extracts are complex and the isolation of bioactive compounds is difficult. Some of the extractants may be useful in yielding less complex mixtures containing the bioactive compounds for subsequent isolation purposes by either extracting non-bioactive components selectively with another extractant subsequently used on the residue.

Snyder and Kirkland (1979) also tested the efficacy of various extractants that differ in polarity and selectivity group such as: hexane, carbon tetrachloride, di-isopropylether, ethyl ether, methylene dichloride, tetrahydrofuran, acetone, ethanol, ethyl acetate, methanol and water. Relatively polar extractants such as ethanol extracted similar concentrations of non-polar compounds.

Rogers (1998) found that many acidic chemical compounds are situated on the surface of leaves and can be dissolved by 1% sodium bicarbonate and recovered by precipitation with hydrochloric acid. Eloff (2000) extracted more compounds with higher antibacterial activity with acetone extractant than with sodium bicarbonate. Therefore, acetone was considered as better solvent than sodium bicarbonate to use when screening a number of plants for antibacterial compounds due to its volality, miscibility with polar and non-polar solvents and its relatively low toxicity to the test organisms.

Traditionally, plant extracts are prepared with water as infusions, decoctions and poultices. Therefore, it would seem unlikely that the traditional healer is able to extract those compounds which are responsible for activity in the non-polar extracts.

2.5.2 Tannin assays

There are several methods used of determining tannin content in plant extracts (Hagerman (1987). These include: colorimetric assay for total phenolics, gravimetric assays, and protein precipitation assays (Cornell 2000; Schofield *et al*. 2001).

2.5.2.1 Colorimetric assay for total phenolics

These assays are mostly used to quantify the total concentration of phenolic compounds in the plant extracts of interest without discriminating between tannins and other phenolics (Hagerman and Butler 1994; Waterman and Mole 1994). This assay is based on the reduction of phosphomolybdic acid by phenols in aqueous alkali.

2.5.2.2 Gravimetric assays

Gravimetric methods are based on precipitation of tannins using ytterbium or PVP. However, these methods are applicable for the determination of soluble tannins and there is low repeatability in plants with low levels of tannins (Reed *et al*. 1985).

2.5.2.3 Protein precipitation assay: radial diffusion

This method is more closely related to the biological effects of tannins since it is based on protein precipitation capacity of tannins. This assay is preferred because it allows determination of tannins in large number of samples with limited laboratory facilities. However, is less useful for quantification than the colorimetric procedures. In radial diffusion plant extracts are placed into wells made on protein embedded agar and allowed to diffuse into the agar. The presence of tannins in plant extracts is indicated by the formation of white precipitation area around the wells and the size of the area reflects the amount of tannins (Hagerman 1987).

2.5.4 Bioassays for antibacterial activity

These are an appraisal of the biological activity of a substance by testing its inhibitory effect on bacterial growth and comparing the result with some accepted standard. Different methods or tests are used:

2.5.4.1 Agar diffusion assays

i. Disc diffusion

The disc diffusion method is widely used for antibacterial activity tests (Kelmanson *et al.* 2000). In this method, one species of bacteria is uniformly swabbed onto a nutrient agar plate while plant extract is applied to sterile filter paper discs that are allowed to dry before being placed onto the seeded top layer of the agar plates (Rasoanaivo and Ratsimamanga-Urveg 1993). Each extract is tested in quadruplicates. The relative effectiveness of a compound is determined by comparing the diameter of the zone of inhibition of bacterial growth around the discs with values in a standard table.

ii. Well diffusion

The agar well diffusion assay uses a similar procedure as disc diffusion assay, except that the extracts are placed into wells made on the hardened agar after the inoculation with standardized bacterial culture. The antimicrobial activity is measured as the diameter (mm) of clear zone of growth inhibition around the well (Hufford *et al.* 1975).

According to Eloff (1998b) most authors use disc or well diffusion assays to determine the antibacterial activity of extracts; however, there are limitations to this method, mainly because of the sensitivity of the test to change in operator technique and also in the subsequent interpretation of zone diameter.

The antimicrobial effect of plant extracts may be inhibited or increased by extrinsic factors or contaminants. The agar type, salt concentration, incubation temperature and molecular size of the antimicrobial component influence results obtained with agar diffusion assays. This technique does not distinguish between bactericidal and bacteriostatic effects and the minimum inhibitory concentration cannot be determined and only detects toxicants that can pass through agar (Eloff 1998b). The technique works well with defined inhibitors, but when examining extracts containing unknown components, there are problems leading to false positive and false negative results. In recent years there has been a move towards a more quantitative method, namely, the measurement of an antimicrobial agent's minimum inhibitory concentration (MIC) (Eloff 2000).

2.5.5 Determination of minimum inhibitory concentration by

i. Agar-dilution method

In this method the plant extract is mixed thoroughly with nutrient agar and allowed to set (Afolayan and Meyer 1997; Grierson and Afolayan 1999). The test organism is streaked in radial patterns on the agar plate. The minimum inhibitory concentration is expressed as the lowest concentration of plant extracts that inhibit bacterial growth. The advantage of Agar-dilution over serial dilution method is that, the concentration of the plant extract to be used should be known at the beginning before streaking the bacteria. However, there are problems with this method; it is difficult to prepare sterile plant extract without the use of autoclaving or other aseptic conditions (Mitscher *et al*. 1972). Furthermore, a larger volume of plant extract is required when using agar-dilution whereas a small amount of volume is needed for serial dilution method.

ii. Serial dilution method for (MIC)

Some researchers used the microtiter plate to detect the presence of antibacterial activity in plant extracts (Eloff 1998b; McGaw *et al*. 2002; Rabe *et al*. 2002; Eloff *et al*. 2005). In this method, plant extract is mixed with nutrient broth in the microplates and then the bacteria are added into the wells. Minimum inhibitory concentration is recorded as the lowest concentration (clear wells) resulting in complete inhibition of bacterial growth. The approximate concentration should be known at the start of the experiment before the appropriate number and amount of dilutions can be made. Serial dilution method is preferable in that it eliminates a lot of the uncertainty and imprecision's involved in making very small concentrations relative to the stock solution, are used instead of making one dilution, in order to finally arrive at the desired concentration. This method is not cost effective but it also allows for small aliquots to be diluted instead of unnecessarily large quantities of materials.

Ethanol, water, acetone and hexane extracts can be used for the bioassay. Antibiotic and extract-free solutions are included as positive and negative controls respectively. An equal volume of nutrient broth and plant extract is added and serially diluted before bacterial culture is added to each well. The culture mixture is incubated for 24 hours.

Iodotetrazolium salt is added to produce a red colour it reduced by bacteria. The inhibition of bacterial growth is visible as a clear mixture.

2.5.6 Bioautography

Bioautography is a very convenient and simple way of testing plant extracts and pure substances for their effects on both human and plant pathogenic microorganisms. It can be employed in the target-directed isolation of active constituents. The antibacterial activity of fractions resulting from each purification stage is tested using the bioautographic assay. An inoculated layer of agar is poured over a developed thin layer chromatography (TLC) plate, and lack of bacterial growth in certain areas identifies the presence and location of antibacterial compounds on the TLC plate. The inhibition of bacterial growth by compounds separated on the TLC plate is visible as white spots against a deep red background (Begue and Kline 1972). Eloff (2001) used bioautographic assay to screened antibacterial compounds from *Sclerocarya birrea*. YFF *et al*. (2002) and Rabe *et al*. (2002) also screened antibacterial compounds from *Pentanisia prunelloides* and *Vernonia colorata*, respectively, where isolation and identification of active compounds were conducted.

2.6 Separation and purification of bioactive compounds

The separation and purification of plant constituents is mainly carried out using one or other, or a combination, of four chromatographic techniques: thin layer chromatography, column chromatography, paper chromatography and high pressure liquid chromatography. The choice of technique or combination depends largely on the solubility, properties and volatilities of the compounds to be separated (Harborne 1984).

2.6.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a fast, relatively cheap and effective method to obtain a characteristic analytical fingerprint of a plant extract (Wagner and Bladt 1995). This technique clearly illustrates differences in chemical composition of plant extracts. The use of TLC to demonstrate the most characteristic constituents of a plant extract or preparation is favoured for its simplicity, rapidity and affordability (McGaw *et al*. 2002).

The resultant chromatography may be interpreted via qualitative (visual) assessment or semi-quantitative analysis of constituents shown (Rios *et al*. 1985; Wagner and Bladt 1995).

Zschocke *et al.* (2000); Kotzé and Eloff (2002); Eloff (2001) successfully used TLC analysis on silica gel to compare the chemical composition of various plant parts of some threatened South African medicinal plants.

The similarity of different extracts from the same plant species can also be assessed in this way and the decision to combine, non-polar and polar extracts, can be made on the basis of identical or similar TLC chromatograms. Qualitative initial screening of extracts should be routinely performed, and the presence of ubiquitous compounds such as plant sterols and certain phenolics can be ascertained at an early stage by running the appropriate standard alongside an extract. In certain cases, classes of compounds may be determined by spraying developed plates with stains that give a color reaction with a particular compounds class. Previous studies found that when vanillin-sulphuric acid spray reagent was applied on TLC-chromatograms more compounds from extract of *Combretum woodii* were visible (Eloff *et al*. 2005). Similar spray reagent was also successfully applied on TLC-chromatograms from several plant extract of *Arctium lappa*, *Tanacetum vulgare*, *Erythrina speciosa*, *Psidium guajava*, *Mikania glomerata*, *Spilanthes acmella*, *Lippia alba*, *Achillea millefolium*, *Piper regnelli*, *Eugenia uniflora*, *Punica granatum*, *Sambucus Canadensis* and *Plantago major* (Holetz *et al*. 2002).

CHAPTER 3

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Plant selection

The following plants, four trees; *Balanites maughamii*, *Breonadia salicina*, *Dombeya rotundifolia*, *Piliostigma thonningii* and one shrub *Hyperacanthus amoenus* were selected for the current study. The selection was based on the information provided by small scale farmers using traditional methods for animal treatment. This information was reported at an Ethnoveterinary medicine workshop held at Giyani, Limpopo Province, South Africa.

3.2 Plant collection

The leaves and bark of selected plants were collected from their natural populations located in the same geographical area of the Basani village in the District Council of Mopani, Limpopo Province (Figure 3.1).

3.3 Plant identification

Collected plants were identified using the herbarium at the University of Limpopo and relevant literature references. Voucher specimen of the plants were prepared and deposited at the University's herbarium (Table 2.1).

3.4 Drying and grinding of plant materials

Collected fresh plant materials were examined for infections and the old, insect-and fungus-infected, leaves and twigs of *B. maughamii*, *D. rotundifolia*, *P. thonningii* and bark of *B. salicina* and *H. amoenus* were removed. Leaves and bark of selected plants were then dried at room temperature ±25ºC for a month. The dry plant materials were ground to pass through a sieve of 1 mm using laboratory grinding mill (SK 100 standard GuBeisen) and stored in airtight bottles until further extraction.
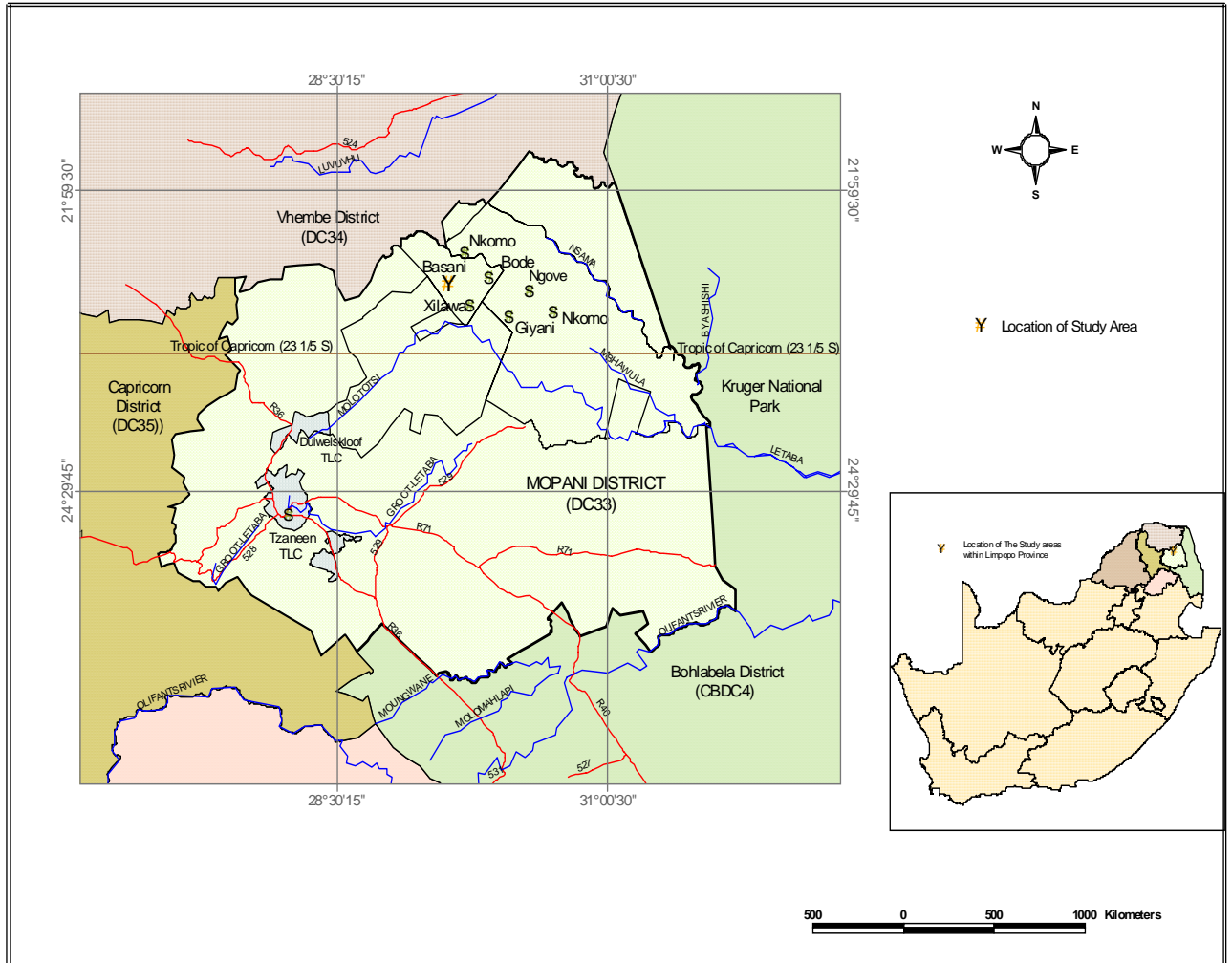
Figure 3.1 Area indicated on the map (Y) shows the collection site of *B. maughamii*, *B. salicina*, *H. amoenus*, *D. rotundifolia* and *P. thonningii*. The S symbol indicates the neighbouring villages around Basani.

3.5 Preparation of crude plant extracts for bioassays

Antimicrobial activity of plant extracts could be affected by the presence of tannins. The effect of tannins on antimicrobial activity of plant extracts could possibly be elucidated by comparative assessment of antibacterial activity of extracts containing tannins with those in which tannins are removed. Therefore, developing a protocol for the preparation of plant extracts suitable for both tannin and antibacterial assays and for complete removal of tannins (*in vitro*) from these extracts was needed.

Acetone is reported to be used commonly as a solvent for preparation of plant extracts for tannin analyses and for determination of antibacterial activities ((70%), Hagerman 1987; FAO/AEIA 2000; (100%) Eloff 1998a; Eloff 2000). Acetone was selected as extraction solvent for preparation of crude extracts of selected plant samples. Preliminary tests, using both 70% and 100% acetone, were performed in this study in order to select a common procedure for preparation of crude extracts suitable for both antimicrobial and tannin assays.

Two grams of each plant material (*B. maughamii*, *B. salicina*, *D. rotundifolia*, *H. amoenus* and *P. thonningii*) were mixed with 50 ml of 70% or 100% acetone into a conical flask. All conical flasks were sonicated in an ultrasonic bath (Bransonic 220) at room temperature for 30 minutes followed by shaking the extract on a Labcon platform horizontal shaker for 1 hour. The extracts were then centrifuged at $1600 \times g$ for 15 minutes. The supernatants were collected and placed in round bottom flasks and evaporated to dryness under reduced pressure on a rotavapor (Optolabor) at 40ºC. The dry yields were dissolved in 70% and 100% acetone respectively to a final concentration of 50 mg ml^{-1}. These crude extracts were used for determination of tannins and screening of antibacterial activity.

3.5.1 Addition of PEG and PVPP to plant extracts for the removal of tannins

Two tannin-binding chemical agents, PEG and PVPP, were used *in-vitro* to test their effectiveness on removal of tannins from the crude plant extracts in order to select the chemical agent and suitable concentration with the highest tannin binding capacity. Polyethylene glycol (PEG) and PVPP (Sigma) were either added to the plant material, from the beginning of the extraction procedure (variant 1) or to the final crude extract (variant 2) to make final concentrations of 0.5% and 1%. In the later, the crude extracts, with and without PEG or PVPP, were incubated on Thermo-mixture at 37 ºC for an hour. Extracts without PEG or PVPP were used as controls for each plant sample.

3.5.2 Determination of tannins

Tannins were determined by radial diffusion assay according to Hagerman (1987). The extract is placed into the wells on solidified agar that is mixed with a protein. When tannins diffuse out of the wells, they form an opaque ring of tannin-protein complex.

3.5.2.1 Preparation of agarose plates

Agarose plates were prepared using 1% agarose solution containing 0.1% bovine serum albumin. Agarose was dissolved in acetate buffer pH 5.0 containing 0.05 M acetic acid and 60 µM ascorbic acid. Agarose solution was heated with continuous stirring until the agarose dissolved (melting point at 86ºC) and the solution became clear. The solution was then cooled to 45ºC in water bath. Bovine serum albumin (0.5 w/v) from Sigma was then added to the heated solution and stirred gently until dissolved completely. To prevent solidification of the agarose, temperature of the solution was kept at 35ºC. The agarose solution (30 ml) was dispensed into sterile petri dishes (90 mm) on a flat surface. Care was taken to prevent formation of bubble while transferring the agarose solution containing bovine serum albumin. The plates were allowed to cool for 30 minutes at room temperature and then closed and sealed with parafilm. The plates were stored for two weeks in refrigerator without losing sensitivity of the assay.

3.5.2.2 Radial diffusion assay

Four uniform wells per plate (6.8 mm in diameter) were made on solidified agarose using cork borer. Seventy five microliter of each plants extracts was placed into the wells using micropipette. The selection of this volume was based on preliminary tests where different volumes of plant extracts were used. The Petri dishes were covered and sealed with parafilm and were incubated on a horizontal platform in an incubator at 30ºC for 96 hours until the reaction was stable. The presence of tannins was indicated by the formation of an opaque zone around the wells. The diameter of this area was measured using veneer caliper. The level of the tannins in each samples were expressed in terms of an average diameter square (cm^2) (Hagerman 1987). Sixteen replicates were made for each sample.

3.6 Determination of antibacterial activity of plant extracts

3.6.1 Bacteria

Four selected bacterial strains, one Gram positive (*Bacillus cereus* ATCC 14579) and three Gram negative (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212), representative of most common pathogens of infectious diseases, were used for antimicrobial tests. Bacterial cultures and McFarland standards were obtained from Davies Diagnostic Company. Nutrient broth was prepared by mixing 0.8 g with 50 ml distilled water into a conical flask and autoclaved for 15 minutes. The sterile nutrient broth was allowed to cool and then a colony from each bacterial strain was inoculated. The bacterial cultures were shaken on a horizontal shaker (150 rpm) for 24 hours. Before the bacterial cultures were used, they were diluted with sterile nutrient broth to a turbidity that matches 0.5 McFarland standard (10^6 Colony Forming Unit (CFU)/ ml^{-1}) (Mulu *et al*. 2004).

3.6.2 Determination of MIC crude plant extract

3.6.2.1 Agar-dilution method

Thirty one gram nutrient broth was mixed with 1 liter distilled water and autoclaved for 15 minutes. The agar solution was allowed to cool to about 60ºC before addition of plant extracts at different concentrations. The agar media containing 0.5, 1.0, 3.0, 4.0, 5.0, 7.0, 9.0, 10.0 and 15.0 mg ml^{-1} respectively, of plant extracts were poured into Petri dishes, swirled carefully until the agar began to set and left overnight on the laminar flow cabinet for the solvents to evaporate (Afolayan and Meyer 1997). Agar plates containing 0.5 ml of acetone were used as control respectively. Each test was replicated four times. On the day of performing the assay, each petri dish was divided into four sections. Each section was inoculated with a different bacterial culture: *B. cereus*, *E. coli*, *E. faecalis* and *P. aeruginosa*. Bacterial cultures were prepared as described 3.6.1. All the Petri dishes were turned up side down, sealed with parafilm and incubated at 37ºC for 72 hours.

3.6.2.2 Serial dilution method

The microplate method of Eloff (1998b) in 96-well microtiter plates was used to determine the minimum inhibitory concentrations (MIC's) of crude plant extracts on bacteria. A hundred microliter nutrient broth (Biolab) as prepared above (3.6.1) was added into each well. A hundred microliter crude plant extract (50 mg ml^{-1}) was added to the first well and mixed with the nutrient broth. Hundred microliters was removed from this well and placed into the next well and so on. The antibiotic tetracycline was used as positive control and acetone as a negative control. A hundred microliter of an actively growing culture of the test organism was added to each of the dilutions. The microplate was sealed and incubated for 24 hours at 37ºC. As an indicator of bacterial growth, 50 μl of 0.2 mg ml^{-1} *p*-iodonitrotetrazolium violet (INT) solution was added to each well after 24 hours and further incubated at 37ºC for 30-120 minutes. The yellowish tetrazolium solution was reduced to a red-coloured product by biological activity and the inhibition of bacterial growth was visible as a clear solution in the well. MIC values were recorded as the lowest concentration of the crude plant extract resulting in complete inhibition of bacterial growth.

3.6.3 Fractionation of crude plant extracts

3.6.3.1 Extraction

Dried, finely ground plant materials (50 g), *B. maughamii, B. salicina, D. rotundifolia, H. amoenus* and *P. thonningii* were extracted with 1 liter methanol on a horizontal platform shaker for overnight. The extracts were filtered through Whatman No.1 filter paper. The residue was washed two times with 200 ml methanol. The resulting filtrate was dried under reduced pressure at 40ºC in a rotavapor (Optolabor). The dried extracts were dissolved in 70% methanol to yield final concentration 50 mg ml^{-1} of crude extract.

3.6.3.2 Solvent-solvent fractionation of crude extracts

Each crude extract (500 ml) was transferred to a separatory funnel and extracted four times with 200 ml hexane. All hexane extracts were collected and combined to form hexane fraction (1). The crude extract was further extracted four times with 200 ml ethyl acetate. All ethyl acetate extracts were combined to form ethyl acetate fraction (2). The crude extract was further extracted four times with 200 ml butanol. All butanol extracts were combined to form butanol fraction (3). The remainder was the aqueous fraction (4). All fractions from different solvents were collected in a round bottom flask and evaporated to dryness under reduced pressure at 40ºC and were dissolved to make a final concentration 50 mg ml^{-1}. The four fractions were used for thin layer chromatography (TLC) and bioautography analyses.

3.6.3.3 Separation of compounds by thin layer chromatography

The fractions (10 µl extract) were applied to a TLC plate (Merck Silica gel 60 F$_{254}$) and the chromatogram developed using hexane: acetone (3: 1) as mobile phase. The thin layer chromatography was performed in duplicate; one for compound detection and the other for bioautography.

3.6.3.4 Detection of separated compounds

The separated compounds were visualized under visible and ultraviolet light (254 and 365) and thereafter stained with vanillin-sulphuric acid spray reagent (0.1 g vanillin, 28 ml methanol and 1 ml sulphuric acid mixed in that order). The reagent was sprayed on the TLC plates, which were thereafter heated for 5 minutes at 110ºC under observation to allow for development of colour changes.

3.6.3.5 Bioautographic assay

Bioautographic assay was performed according to Slusarenko *et al*. (1989) in order to locate the active compounds of the extracts. *Pseudomonas aeruginosa* was used as test organism. Nutrient broth and bacteria were prepared as described above (3.6.1). The bacteria were centrifuged at $1600 \times$ g for 10 minutes and the supernatant decanted. The pellet was diluted with sterile nutrient broth to a turbidity that matches 0.5 McFarland standard (10^6 Colony Forming Unit (CFU)/ ml^{-1}) (Mulu *et al*. 2004) and this was sprayed onto the thin layer chromatograms. The plates were then placed in a chamber at 100% humidity. The chamber was left in the incubator at 37ºC overnight to allow the bacteria to grow. The following day, a 2 mg ml^{-1}solution of INT (Sigma) was sprayed onto the bacteria covered plate which was then placed back in the oven in 100% humidity for 30 minutes. The INT stained the plates dark red making it easy to observe where bacterial growth had been inhibited. The bands that remained in a clear zone indicated compounds with antibacterial activity. Active compounds were identified by comparing the zones of growth inhibition to a duplicate TLC plate developed under identical conditions.

3.7 Statistical Analysis

Analysis of variance (ANOVA) was carried out using the General linear model procedure of SAS (statistical analysis system) (1997). The least square means (LSM) was used to compare differences between the means of diameters, at a 5% of significance ($P < 0.05$).

CHAPTER 4

RESULTS

4.1 Identification of plant materials

Plant species namely, *B. maughamii*, *B. salicina*, *D. rotundifolia*, *H. amoenus* and *P. thonningii* were identified for the current study (Figure 2.1-2.5). The specimens were deposited at University of Limpopo herbarium (Table 2.1).

4.2 Tannins in plant extracts

4.2.1 Tannins in 70% acetone plant extracts

Table 4.1 shows the tannin levels in 70% acetone extracts from the five plants in the presence and absence (controls) of tannin binding reagents (PEG, PVPP) at concentration 0.5% and 1.0 % (w/v) (variant 1). Extract of *P. thonningii* showed the highest levels of tannins 3.38 cm^2 among all plant extracts, while no tannins were recorded in 70% acetone extract of *B. maughamii*. There were no significant differences (P < 0.05) in tannin levels between *B. salicina*, *D. rotundifolia* and *H. amoenus* (Table 4.1).

4.2.1.1 Effect of PEG and PVPP on binding tannins from 70% acetone extract when added at the beginning of the extraction (variant 1)

Addition of PEG and PVPP to the extraction media (variant 1) resulted in reduction of tannins in all crude plant extracts with PEG being more effective than PVPP. The percentage of tannin removed depended on the type and the concentration of the precipitating agent used. In general, 1% PEG appear to remove higher percentages of tannins in all plant extracts, in comparison to 0.5% PEG and to 0.5 and 1% PVPP (24.8 and 49.4) respectively (Table 4.1). The highest percentage of tannin removal (68.4) was achieved with 1% PEG in *P. thonningii*. One percent PVPP resulted in lower percentage (49.4) of tannin removal for this extract. Similar results were observed for *B. salicina* and *D. rotundifolia* where addition of 1% PVPP resulted in lower percentage (15.0 and 13.1) of tannin removal. In particular, 0.5% PVPP followed by 0.5% PEG were less effective on tannin removal from all 70% acetone plant extracts than 1% PVPP and 1% PEG.

Table 4.1 Tannins in 70% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction (variant 1)

Plant species	PEG and PVPP (%)	Tannin level (cm^2)			
		PEG		PVPP	
		Tannin level (cm^2)	% of tannins removed	Tannin level (cm^2)	% of tannins removed
B. m	0.0	0.00^f	0.00	0.00^h	0.00
	0.5	0.00^f	0.00	0.00^h	0.00
	1.0	0.00^f	0.00	0.00^h	0.00
B. s	0.0	2.12^b	0.00	2.12^c	0.00
	0.5	1.54^c	27.4	1.81^d	15.0
	1.0	1.06^e	50.0	1.56^e	26.4
D. r	0.0	2.06^b	0.00	2.06^c	0.00
	0.5	1.56^c	24.3	1.79^d	13.1
	1.0	1.20^d	41.7	1.59^e	22.8

B. m = Balanites maughamii, B. s = Breonadia salicina, D. r = Dombeya rotundifolia

Column denoted by the same superscripts are not significantly different (P < 0.05)

Table 4.1(*continue*) Tannins in 70% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction (variant 1)

Plant species	PEG and PVPP (%)	Tannin level (cm^2)				
		PEG		PVPP		
		Tannin level (cm^2)	% of tannins removed	Tannin level (cm^2)	% of tannins removed	
H. a	0.0	2.07[b]	0.00	2.07[c]	0.00	
	0.5	1.58[c]	23.7	1.29[f]	37.7	
	1.0	1.09[e]	47.3	1.17[g]	43.5	
P. t	0.0	3.38[a]	0.00	3.38[a]	0.00	
	0.5	1.24[d]	63.3	2.54[b]	24.8	
	1.0	1.07[e]	68.3	1.71[d]	49.4	

H. a = Hyperacanthus amoenus, P. t = Piliostigma thonningii

Column denoted by the same superscripts are not significantly different (P < 0.05)

4.2.2 Tannins in 100% acetone extracts

Crude plant extracts prepared with 100% acetone showed similar pattern of variation of tannins between plant samples compared to 70% acetone extracts except for *B. maughamii* which showed no presence of tannins (Table 4.2). However, higher tannins levels were recorded in all plant samples extracted with 100% acetone than with 70%. In *P. thonningii* and *B. salicina* plant samples the diameter square of the area of precipitation which reflects the level of tannins was increased from 3.38 to 4.29 and from 2.12 to 3.43 respectively (Table 4.3). Unlike in 70% acetone extracts, tannin levels in 100% acetone extracts from all plant samples differed significantly (Table 4.2).

4.2.2.1 Effect of PEG and PVPP on binding tannins from 100% acetone extracts when added at the beginning of the extraction (variant 1)

Both PEG and PVPP were more effective in the removal of tannins from 100% acetone extracts (up to 84% for *P. thonningii*) than from 70% acetone extracts (up to 64% for *P. thonningii*). Similarly to the 70% acetone extracts 1% PEG proved to be more effective than 0.5% PEG, 0.5% and 1% PVPP in reducing tannin levels in the crude plant extract resulting in the highest percentage of tannin removal up to 84% for *P. thonningii* (Table 4.2).

Table 4.2 Tannins in 100% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction (variant 1)

Plant species	PEG and PVPP (%)	Tannin level (cm^2)			
		PEG		PVPP	
		Tannin level (cm^2)	% of tannins removed	Tannin level (cm^2)	% of tannins removed
B. m	0.0	0.00[j]	0.00	0.00[j]	0.00
	0.5	0.00[j]	0.00	0.00[j]	0.00
	1.0	0.00[j]	0.00	0.00[j]	0.00
B. s	0.0	3.43[b]	0.00	3.43[b]	0.00
	0.5	1.06[f]	69.1	2.17[e]	63.3
	1.0	0.94[g]	72.6	1.87[f]	54.5
D. r	0.0	2.41[d]	0.00	2.41[d]	0.00
	0.5	0.93[gh]	61.4	1.83[g]	24.1
	1.0	0.71[i]	70.5	1.04[i]	56.8

Column denoted by the same superscripts are not significantly different at P < 0.05

Table 4.2(*continue*) Tannins in 100% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction (variant 1)

Plant species	PEG and PVPP (%)	Tannin level (cm^2)			
		PEG		PVPP	
		Tannin level (cm^2)	% of tannins removed	Tannin level (cm^2)	% of tannins removed
H. a	0.0	2.67[c]	0.00	2.67[c]	0.00
	0.5	0.91[gh]	41.2	1.84[fg]	31.1
	1.0	1.57[e]	66.0	1.56[h]	41.6
P. t	0.0	4.29[a]	0.00	4.29[a]	*0.00*
	0.5	0.90[h]	79.0	2.42[d]	43.6
	1.0	0.69[i]	84.0	1.53[h]	64.3

Column denoted by the same superscripts are not significantly different at P < 0.05

Table 4.3 Tannins in 70% and 100% acetone extracts from selected plant samples in the absence of PEG and PVPP (controls)

Solvent	Plant species				
	B. m	*B. s*	*D. r*	*H. a*	*P. t*
70%	0.00^a	2.12^b	2.06^b	2.07^b	3.38^b
100%	0.00^a	3.43^a	2.41^a	2.67^a	4.29^a

Column denoted by the same superscripts are not significantly different at P < 0.05

4.2.2.2 Effect of PEG on binding tannins from 100% acetone extract when added to the final extracts (variant 2)

Table 4.4 shows tannin levels in plant extracts with or without 1% PEG when added to the final crude plant extract instead to the plant material from the beginning of the extraction procedure as in 4.2.2. Addition of 1% PEG to the final extract resulted in 100% removal of tannins in all plant samples since no precipitation areas around the wells were recorded (Fig 4.1).

Table 4.4 Tannins in 100% acetone extracts from selected plant samples in the presence and absence of PEG added to the final extraction (variant 2)

Plant Species	PEG (%)	Tannin level (cm^2) PEG	
		Tannin level (cm^2)	% of tannins removed
B. m	0.0	0.00[e]	0.00
	1.0	0.00[e]	0.00
B. s	0.0	3.43[b]	0.00
	1.0	0.00[e]	100
D. r	0.0	2.41[d]	0.00
	1.0	0.00[e]	100
H. a	0.0	2.67[c]	0.00
	1.0	0.00e	100
P. t	0.0	4.30[a]	0.00
	1.0	0.00[e]	100

Column denoted by the same superscripts are not significantly different at P < 0.05

Table 4.5 Comparison of tannins in 70% and 100% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction

Plant species	PEG and PVPP (%)	Tannin level (cm^2)							
		Tannin level (cm^2)		% of tannins removed		Tannin level (cm^2)		% of tannins removed	
		PEG	PEG	PEG	PEG	PVPP	PVPP	PVPP	PVPP
		70%	100%	70%	100%	70%	100%	70%	100%
B. m	0.0	0.00^f	0.00^j	0.00	0.00	0.00^h	0.00^j	0.00	0.00
	0.5	0.00^f	0.00^j	0.00	0.00	0.00^h	0.00^j	0.00	0.00
	1.0	0.00^f	0.00^j	0.00	0.00	0.00^h	0.00^j	0.00	0.00
B. s	0.0	2.12^b	3.43^b	0.00	0.00	2.12^c	3.43^b	0.00	0.00
	0.5	1.54^c	1.06^f	27.4	69.1	1.81^d	2.17^e	15.0	63.3
	1.0	1.06^e	0.94^g	50.0	73.0	1.56^e	1.87^f	26.4	54.5
D. r	0.0	2.06^b	2.41^d	0.00	0.00	2.06^c	2.41^d	0.00	0.00
	0.5	1.56^c	0.93^{gh}	24.3	61.4	1.79^d	1.83^g	13.1	24.1
	1.0	1.20^d	0.71^i	42.0	70.5	1.59^e	1.04^i	22.8	57.0

Column denoted by the same superscripts are not significantly different at P < 0.05

Table 4.5 (*continue*) Tannins in 70% and 100% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction

Plant species	PEG and PVPP (%)	Tannin level (cm^2)							
		Tannin level (cm^2)		% of tannins removed		Tannin level (cm^2)		% of tannins removed	
		PEG	PVPP	PEG	PVPP	PEG	PVPP	PEG	PVPP
		70%	100%	70%	100%	70%	100%	70%	100%
H. a	0.0	2.07[b]	2.67[c]	0.00	0.00	2.07[c]	2.67[c]	0.00	0.00
	0.5	1.58[c]	0.91[gh]	24.0	41.2	1.29[f]	1.84[fg]	38.0	31.1
	1.0	1.09[e]	1.57[e]	47.3	66.0	1.17[g]	1.56[h]	43.5	42.0
P. t	0.0	3.38[a]	4.29[a]	0.00	0.00	3.38[a]	4.29[a]	0.00	0.00
	0.5	1.24[d]	0.90[h]	63.3	79.0	2.54[b]	2.42[d]	25.0	44.0
	1.0	1.07[e]	0.69[i]	68.3	84.0	1.71[d]	1.53[h]	49.4	64.3

Column denoted by the same superscripts are not significantly different at P < 0.05
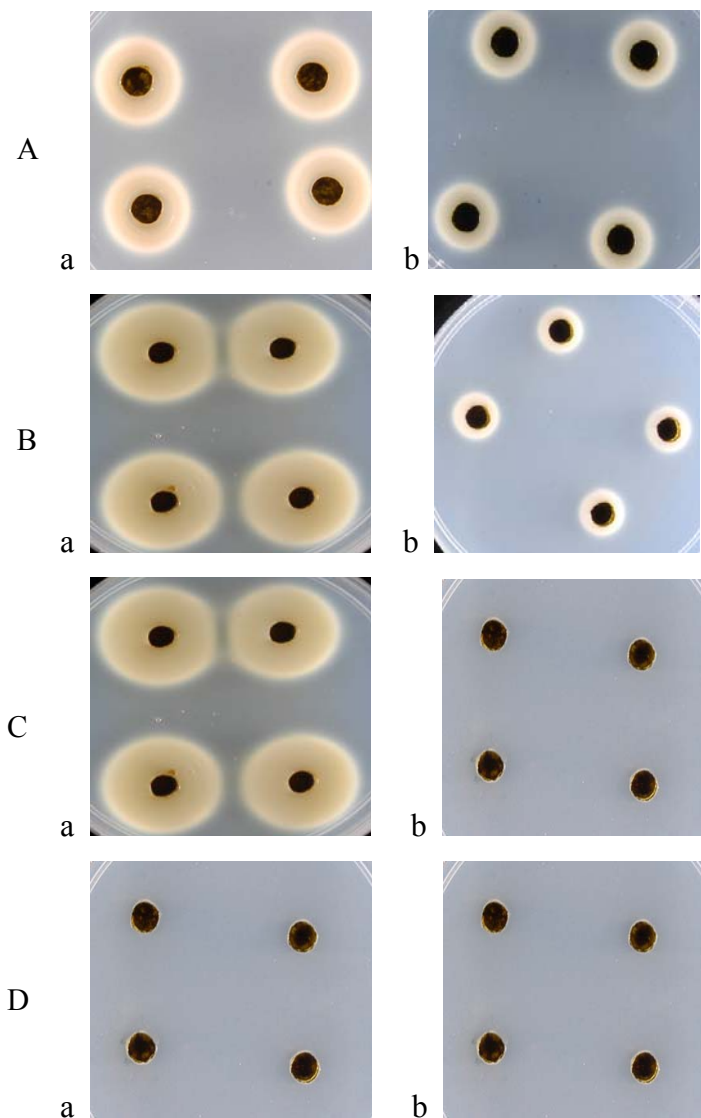
Figure 4.1 (A) and (B) tannins in 70% and 100% acetone extract of *P. thonningii* added to the plant material (variant 1), respectively, (C) tannins in 100% acetone extract of *P. thonningii* added to the final crude extract (variant 2), (D) tannins in 100% acetone extract of *B. maughamii* added to the plant material (variant 1). (a) without PEG (control) and (b) with 1% PEG. The white areas around the wells indicate presence of tannins.

4.2.6 Minimum inhibitory concentration of plant extracts

4.2.6.1 Minimum inhibitory concentration of 100% acetone extracts (agar-dilution method)

Table 4.6 shows the antibacterial activity of 100% acetone extracts of five plant samples (*B. maughamii, B. salicina, D. rotundifolia, H. amoenus* and *P. thonningii*) against the four test micro-organism (*B. cereus, E. coli, E. faecalis* and *P. aeruginosa*) using the agar-dilution method. The extract of *B. maughamii* and *D. rotundifolia* yielded the same activity against *B. cereus* with MIC value of 0.5 mg ml^{-1}. There were no differences in the antibacterial activities against *E. coli, E. faecalis* and *P. aeruginosa* for which MIC values were two-fold higher (1.0 mg ml^{-1}) than *B. cereus*. In contrast, the extract of *B. salicina* showed lower activity against all bacteria strains with MIC values of 4.0 mg ml^{-1} against *B. cereus* and 5 mg ml^{-1} against *E. coli, E. faecalis* and *P. aeruginosa*. The extract of *H. amoenus* demonstrated antibacterial activity (MIC 3.0 mg ml^{-1}) against *B. cereus* and against *E. coli, E. faecalis* and *P. aeruginosa* (MIC 4.0 mg ml^{-1}). The extract of *P. thonningii* showed the highest antibacterial activity against *B. cereus* (MIC 0.5 mg ml^{-1}) among all plants tested.

4.2.6.2 Effect of PEG on MIC of 100% acetone extracts (agar-dilution method)

Addition of 1% PEG to 100% acetone extract of five plant species (*B. maughamii, B. salicina, D. rotundifolia, H. amoenus* and *P. thonningii*) increased the MIC values for all plant extracts (5.0-15.0 mg ml^{-1}). The extracts had lower antibacterial activity when compared to the MIC values obtained for controls as described in 4.2.6.1. The highest percentage reduction (95%) of antibacterial activity with 1% PEG in both extracts of *B. maughamii* and *D. rotunfifolia* was achieved against *B. cereus*. On the other hand, the lowest percentage reduction (20.0%) of antibacterial activity for *H. amoenus* was achieved against *E. faecalis* and *P. aeruginosa* (Table 4.6).

Table 4.6 Minimum inhibitory concentration of 100% acetone extracts in the presence and absence of PEG (agar-dilution method)

Plant species	Plant Part	PEG (%)	MIC (mg ml^{-1})							
			B. cereus		*E. coli*		*E. faecalis*		*P. aeruginosa*	
			MIC	% increase in MIC	MIC	% increase in MIC	MIC	% increase in MIC	MIC	% increase in MIC
B. m	LF	0.0	0.5	0.00	1.0	0.00	1.0	0.00	1.0	0.00
		1.0	10.0	95.0	15.0	93.3	15.0	93.3	15.0	93.3
B. s	BK	0.0	4.0	0.00	5.0	0.00	5.0	0.00	5.0	0.00
		1.0	10.0	60.0	10.0	50.0	10.0	50.0	10.0	50.0
D. r	LF	0.0	0.5	0.00	1.0	0.00	1.0	0.00	1.0	0.00
		1.0	10.0	95.0	10.0	90.0	10.0	90.0	5.0	80.0
H. a	BK	0.0	3.0	0.00	4.0	0.00	4.0	0.00	4.0	0.00
		1.0	10.0	70.0	10.0	60.0	5.0	20.0	5.0	20.0
P. t	LF	0.0	0.5	0.00	1.0	0.00	0.5	0.00	0.5	0.00
		1.0	5.0	90.0	10.0	90.0	5.0	90.0	5.0	90.0

B. cereus = Bacillus cereus, E. coli = Escherichia coli, E. faecalis = Enterococcus faecalis, P. aeruginosa = Pseudomonas aeruginosa. Plant part: LF = leaf, BK = bark

4.2.7.1 Minimum inhibitory concentration of 100% acetone extracts (serial dilution method)

Table 4.7 shows the screening for the antibacterial activity of the 100% acetone extracts against the four bacteria using the serial dilution method. The extract of *B. maughamii* showed activity against *B. cereus*, *E. coli* and *P. aeruginosa* with MIC values ranging from 0.78 to 1.56 mg ml^{-1} while greater activity was obtained against *E. faecalis* with an MIC value of 0.195 mg ml^{-1}. The extract of *B. salicin*a showed the same activity against *B. cereus*, *E. faecalis* and *P. aeruginosa* with an MIC value of 0.78 mg ml^{-1} while the extract of *D. rotundifolia* showed the same MIC value (0.39 mg ml^{-1}) against *B. cereus*, *E. faecalis* and *P. aeruginosa* except for *E. coli* with MIC value of 0.78 mg ml^{-1}. The extract of *H. amoenus* showed the same activity against *B. cereus* and *E. coli* (MIC 1.56 mg ml^{-1}) and the highest activities were observed against *E. faecalis* and *P. aeruginosa* with MIC values ranging between 0.39 and 0.78 mg ml^{-1}. The *P. thonningii* extract inhibited the growth of *B. cereus*, *E. coli* and *E. faecalis* with MIC values ranging from 0.39 to 1.56 mg ml^{-1}. A higher activity was observed against *P. aeruginosa* with an MIC value of 0.195 mg ml^{-1}.

4.2.7.2 Effect of PEG on MIC of 100% acetone extracts (serial dilution method)

The minimum inhibitory concentration values of the 100% acetone extract with addition of 1% PEG against the four test organisms (*B. cereus*, *E. coli*, *E. faecalis* and *P. aeruginosa*) are summarized in Table 4.7. Addition of 1% PEG to the acetone extracts of plant material increased the MIC values which imply a reduction of antibacterial activity. The highest percentage reduction (97.8%) of antibacterial activity was achieved for *B. maughamii* against *E. faecalis*. On the other hand the lowest percentage reduction (50%) of antibacterial activity for extracts of *B. maughamii*, *B. salicina* and *P. thonningii* was against *E. coli* and *P. aeruginosa*.

In this study, tetracycline hydrochloride (an antibiotic) showed a higher antibacterial activity (MIC values ranging between 0.01 and 0.03 mg ml^{-1}) against four test bacteria as compared to five plant extracts.

Table 4.7 Minimum inhibitory concentration of 100% acetone extracts in the presence and absence of PEG (serial dilution method)

Plant species	Plant part	PEG (%)	MIC (mg ml^{-1})							
			B. cereus		E. coli		E. faecalis		P. aeruginosa	
			MIC	% increase in MIC	MIC	% increase in MIC	MIC	% increase in MIC	MIC	% increase in MIC
B. m	LF	0.0	0.78	0.00	0.78	0.00	0.195	0.00	1.56	0.00
		1.0	6.25	87.5	12.5	93.8	3.13	97.8	3.13	50.0
B. s	BK	0.0	0.78	0.00	1.56	0.00	0.78	0.00	0.78	0.00
		1.0	6.25	87.5	3.13	50.0	6.25	87.5	3.13	75.1
D. r	LF	0.0	0.39	0.00	0.78	0.00	0.39	0.00	0.39	0.00
		1.0	3.13	87.5	3.13	75.1	3.13	87.5	1.56	75.0
H. a	BK	0.0	1.56	0.00	1.56	0.00	0.39	0.00	0.78	0.00
		1.0	6.25	75.0	12.5	87.5	3.13	87.5	3.13	75.1
P. t	LF	0.0	0.39	0.00	0.78	0.00	1.56	0.00	0.195	0.00
		1.0	1.56	75.0	1.56	50.0	6.25	75.0	3.13	93.8
Tetracycline			0.01		0.02		0.03		0.02	

4.3 Thin layer chromatography and bioautography

In *B. maughamii*, two spots were visualized on thin layer chromatography (TLC) in the ethyl acetate fraction (R_f 0.33 and 0.37) while no spots were visible in the other fractions. The second spot with an R_f value of 0.37 showed antibacterial activity. The extract of *B. salicina* showed five spots in the crude methanol extract (R_f 0.09, 0.11, 0.25, 0.33 and 0.40), six in the hexane fraction (R_f 0.05, 0.09, 0.11, 0.23, 0.24 and 0.53), four in the ethyl acetate fraction (R_f 0.09, 0.11, 0.38 and 0.56) and one in the butanol fraction (R_f 0.15). The bioautography assay showed the following spots: crude methanol extract (R_f 0.09 and 0.11), hexane (R_f 0.11, 0.23, 0.24 and 0.53), butanol (R_f 0.15) and the ethyl acetate fractions (R_f 0.09 and 0.11), all active against *P. aeruginosa*.

In *D. rotundifolia*, five spots were visible in the crude methanol extract (R_f 0.11, 0.41, 0.50, 0.57 and 0.84); three in the ethyl acetate fraction (R_f 0.16, 0.43 and 0.64) and no spots were separated in the hexane, butanol and aqueous fractions. Spots with antibacterial activity were visible in the crude methanol extract (R_f 0.11) and ethyl acetate fraction (R_f 0.16). Low activity was observed in the butanol fraction. In the extract of *H. amoenus* four spots were visible in the crude methanol (R_f 0.48, 0.52, 0.59 and 0.71), four in the hexane fraction (R_f 0.12, 0.14, 0.16 and 0.76). Spots with antibacterial activity were observed in the crude methanol extract (R_f 0.48), hexane fraction (R_f 0.12, 0.14, 0.16 and 0.76), butanol (R_f 0.12) and ethyl acetate fractions (R_f 0.12 and 0.14) while low activity was observed in the hexane fraction (R_f 0.12, 0.14, 0.16 and 0.76). In *P. thonningii* six spots were visible in the crude methanol extract (R_f 0.29, 0.34, 0.41, 0.61, 0.66 and 0.75), in butanol fraction (R_f 0.40, 0.59 and 0.81) and another three in the ethyl acetate fraction (R_f 0.59, 0.81 and 0.76). Higher antibacterial activity was observed in the ethyl acetate fraction (R_f 0.59) and low activity in the butanol fraction (R_f 0.40, 0.59).
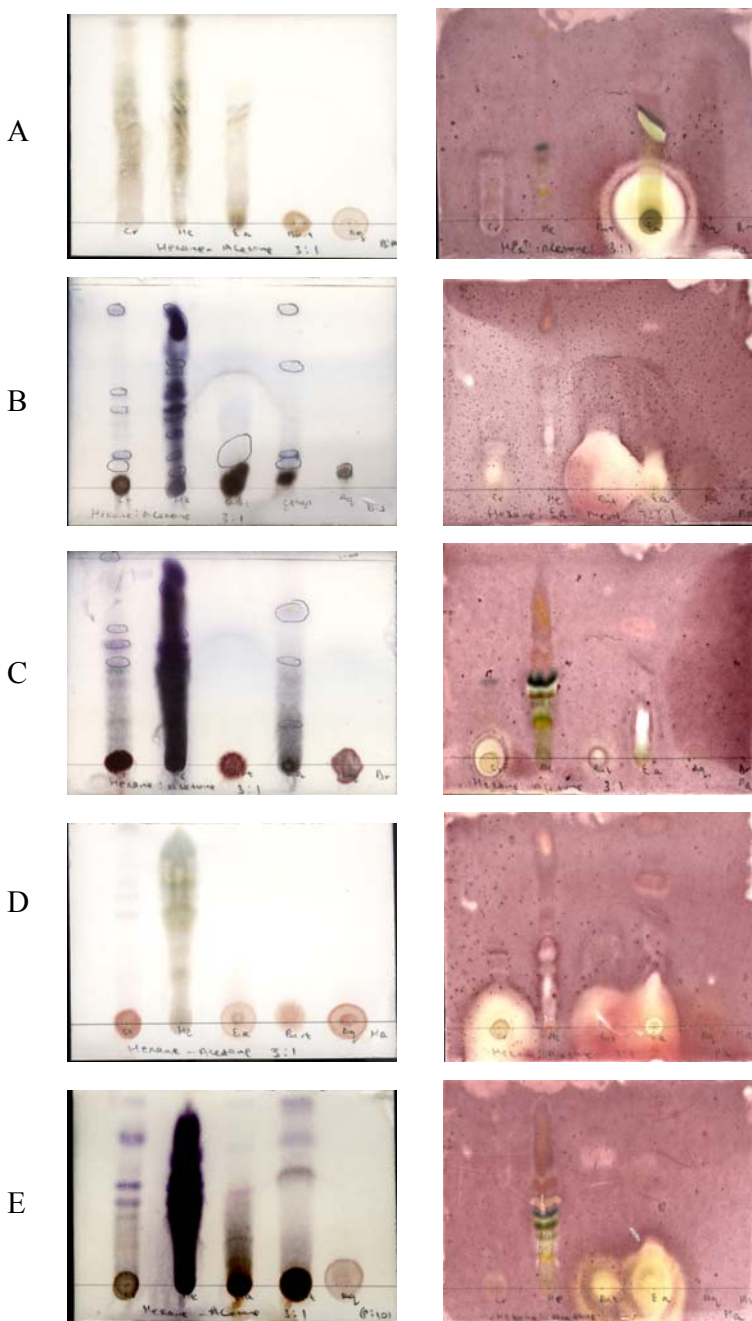
Figure 4.2 Left and right shows TLC and bioautography of (A) *B. maughamii*, (B) *B. salicina*, (C) *D. rotundifolia*, (D) *H. amoenus* and (E) *P. thonningii*, respectively. TLC chromatogram sprayed with vanillin-sulphuric acid spray reagent. The bioautography sprayed with *P. aeruginosa* as test organism. Fractions of Cr = Crude methanol, He = hexane, Ea = ethyl acetate, But = butanol and Aq = aqueous were used.

CHAPTER 5
DISCUSSION

5. DISCUSSION

5.1 Tannins

In this study, radial diffusion assay was used for screening the level of tannins in the five plant extracts (*B. maughamii*, *B. salicina*, *D. rotundifolia*, *H. amoenus* and *P. thonningii*) since this method is rapid, easy and showed good repeatability of results which confirms the report by (Hagerman 1987).

Acetone was selected as a solvent to extract tannins from all plant samples in this study, since it is reported to be used for preparation of plant extracts for both, tannin and antibacterial, bioassays (Hagerman 1987; Eloff 2000). However, 100% acetone is mostly used for preparation of plant extracts for antibacterial tests while 70% acetone and 50% methanol are used for extracting tannins from plant materials (Hagerman 1987; FAO/IAEA 2000) since 100% acetone is considered to inhibit interaction between tannins and proteins (Hagerman 1987; Hagerman and Robbins 1987; FAO/IAEA 2000; Cornell, 2000). Some authors have found that aqueous acetone was better solvent for extracting condensed tannins than aqueous methanol but condensed tannins were less stable in aqueous acetone than in aqueous methanol (Cork and Krockenberger 1991). Aqueous acetone is also used to extract hydrolysable tannins, but no quantitative estimates of recovery of tannins are reported (Salminen 2003).

Comparative assessment of tannins in both, 70% aqueous acetone and 100% acetone, extracts was done in this study in order to select appropriate extraction procedure suitable for both assays. Our results showed higher level of tannins in 100% acetone extracts from all plant samples than in 70% acetone extracts which is in contrast to other reports mentioned above. Based on these results and considering the reports on plant extracts used for antibacterial assays, 100% acetone was selected as an extraction solvent for preparation of all plant extracts in this study since it appeared to be suitable for both, tannin and antibacterial, assays.

The highest tannin levels in both, 70% and 100% acetone, extracts was recorded for *P. thonningii* while no tannins were detected by radial diffusion assay in *B. maughamii*. This could be due to the presence of insoluble tannins since in many plants more than 50% of tannins are reported to be not extractable due to insolubility (Cornell 2000).

Air dried plant materials at room temperature (± 25˚C) were used in this study for preparation of acetone extracts from all plant samples as reported by other studies on antibacterial activity of plant samples (Eloff 2000). Salminem (2003) discovered that freezing and vacuum drying of plant material provide more reliable results for tannins than air or oven dried material. Although freezing the sample was reported by FAO/IAEA (2000) to be better option, thawing of the plant material could create problem due to rupture of cell membranes thus leading to changes in phenolics (FAO/IAEA 2000). Drying plant samples above 40˚C have been reported to reduce the solubility of tannins and their ability to complex proteins due to tannin polimerisation, thus resulting in a lower number of free hydroxyl groups available for bonding the proteins (Cornell 2000). Drying of plant tissues at ambient or at elevated temperature is considered more convenient than freezing, although less tannins can be extracted from samples dried at elevated temperature (Bate-Smith 1975; Price *et al*. 1979). Since the aim of this study was to record the tannins available in crude plant extracts used for testing antibacterial activities in selected plants, the air drying procedure of plant materials appeared to be relevant and was adopted for the purpose of this study.

Two tannin-binding reagents (PEG and PVPP) at different concentrations (0.5 and 1%) were used to remove tannins from 70% and 100% acetone extracts of *B. maughamii*, *B. salicina*, *D. rotundifolia*, *H. amoenus* and *P. thonningii*. Comparative assessment of the effect of the two selected precipitating agents, PEG and PVPP, at two different concentrations (0.5% and 1%) revealed that 0.5% of both, PEG and PVPP, removed less tannins in both 70% and 100% acetone extracts (variant 1) as compared to 1% of PEG and PVPP with PEG being more effective than PVPP. Similar results have been obtained by Makkar *et al*. (1995) who used PEG to bind tannins in leaf material of *Acioa barteri*,

Dichostachys cinerea, *Guiera senegalensis* and *Piliostigma reticulatum*. Polyethylene glycol is reported to have higher binding affinity to proteins as compared to PVPP (Makkar 2003). Addition of PEG results in the formation of PEG-tannin complexes which inactivates tannins. Polyethylene glycol is reported to react preferentially with condensed tannins and can prevent the formation of tannin-protein complexes (Jones and Mangan 1977). The addition of 1% PEG to plant samples, at the beginning of the extraction procedure (variant 1), in this study resulted in the highest percentage of tannin removal, up to 84%, and therefore, it was selected as a precipitating agent for binding tannins.

Further tests revealed that the addition of 1% PEG to the final crude extract (50 mg ml^{-1}) (variant 2), instead at the beginning of the extraction (variant 1), and incubation at 37˚C can result in 100% tannin removal as detected by the radial diffusion assay. Previous studies reported that PEG melts at about 56˚C and at room temperature the solubility is approximately 50% (Vasa 2006). It was observed in this study that PEG dissolves at 37˚C while it remains insoluble at room temperature. This result suggests that the solubility of PEG could influence its binding capacity for tannins. Additional advantage of this procedure (variant 2) is that it requires less PEG since the binding agent is added to smaller volumes of plant extracts than in variant 1 where bigger volumes of solvents were added to plant materials from the beginning of the extraction.

From this study on tannins, it appears that the addition of 1% PEG to the final extract seems to be the most suitable option for complete removal of tannins from all, tannin-containing, plant samples and therefore, 1% PEG was selected as tannin precipitating agent for crude plant extracts used for antibacterial assays.

5.2 Antibacterial activity

There are different methods or technique that can be used to determine antibacterial activity of plant extracts. Some of these are: disc diffusion, agar well diffusion, agar-dilution and serial dilution method (Kelmanson *et al*. 2000; Hufford *et al*. 1975; Afolayan and Meyer 1997; Eloff 1998b). The last one is believed to be more sensitive than the others (Eloff 1998b).

In this study two methods, serial dilution and agar-dilution were used to determine the antibacterial activity of five plant extracts (*B. maughamii*, *B. salicina*, *D. rotundifolia*, *H. amoenus* and *P. thonningii*) using four test organisms (*B. cereus*, *E. coli*, *E. faecalis* and *P. aeruginosa*).

5.2.1 Agar-dilution method

In agar-dilution method, each plant extract was mixed with nutrient agar and the complete suppression of bacterial growth was required for an extract to be declared active. This method is convenient and suitable for the small laboratory. However, the agar-dilution method has the disadvantage of contamination and sterility of the extract is necessary in order to achieve accurate results (Mitscher *et al*. 1972).

The extract of *B. maughamii* (without PEG) gave the lowest MIC value (0.5 mg ml^{-1}) and no differences between its activity against *E. coli*, *E. faecalis* and *P. aeruginosa* were noted (where the MIC was 1.0 mg ml^{-1}). To the best of our knowledge, no findings were reported previously in the literature on the antibacterial activity of *B. maughamii*. However, addition of (1%) PEG to the plant extract resulted in an increase in MIC values (from 0.5 to 10.0 mg ml^{-1} against *B. cereus*), indicating that the extract has low activity against Gram-positive and Gram-negative bacteria. Note that no tannins were recorded in this extract (Section 5.1). Surprisingly, this observation on *B. maughamii* showed similar trends as those plant extracts containing tannins as discussed above. The antibacterial activity of this plant extract could be as a result of other secondary metabolites than

69

tannins. There is a possibility that PEG may bind to some secondary metabolites (bioactive compounds), except tannins. For example, previous studies showed that *Balanites* species contain numerous steroidal glycosides derived from diosgenin and structurally related sapogenins. Activities that have been ascribed to saponins include anti-fungal, anti-viral, spermicidal and molluscicidal effects (Pretorius *et al*. 1988).

Extracts of *B. salicina* showed antibacterial activity against *B. cereus* and no differences were observed between the activities obtained against *E. coli*, *E. faecalis* and *P. aeruginosa*. The activity was observed at a high concentration of 5000 μg ml^{-1} while some researchers found that pure extracts of *Helichrysum aureonitens* showed antibacterial activity against Gram-positive bacteria at lower concentrations, viz., 100 and 500 μg ml^{-1} and inhibitory activity was not detected against Gram-negative bacteria (Afolayan and Meyer 1997). Notwithstanding, the current findings showed that plant samples containing tannins showed low activity (MIC 10.0 mg ml^{-1}) against Gram-positive and Gram-negative bacteria. The extract of *D. rotundifolia* inhibited the growth of the Gram-positive and no differences were observed against the Gram-negative bacteria. The highest MIC value obtained in this study was 500 μg ml^{-1} (Table 4.6), indicating that the extract is active. However, Reid *et al*. (2001) used the disc diffusion assay for screening of antibacterial activity of *D. rotundifolia* and found that Gram-negative bacteria were more resistant than Gram-positive bacteria. Antibacterial activity (MIC 0.40 and 0.12 mg ml^{-1} against *B. subtilis*) was previously detected in leaf and shoot material from *D. rotundifolia* (McGaw 2000; Reid *et al*. 2001). Plant extracts containing 1% (PEG) showed the same inhibitory activity against *B. cereus*, *E. coli* and *E. faecalis* except for *P. aeruginosa*. In general, addition of 1% PEG to the plant material reduced the antibacterial activity.

The MIC of *H. amoenus* extract was determined against Gram-positive and Gram-negative bacteria. The extract of *H. amoenus* showed lower antibacterial activity against *E. coli*, *E. faecalis* and *P. aeruginosa* (all Gram-negative) as compared to *B. cereus* (Gram-positive). Therefore, the results obtained are by no means surprising since Gram-

negative are more resistant than the Gram-positive bacteria as confirmed by Martin (1995); Paz *et al*. (1995); Vlietinck *et al*. (1995). The MIC of plant extracts in the presence of 1% PEG was higher; this implies that crude extract would probably not be useful for isolation of pharmaceutically active compounds (Rios *et al*. 1988). Reasons for the relatively high MIC values in the presence of PEG could be that the extracts tested are still in an impure form, or that the active compounds are present in very low concentrations (Rabe and Van Staden 1997).

Evaluation of the antibacterial activity of the *P. thonningii* extract showed the same inhibitory effect against *B. cereus*, *E. faecalis* and *P. aeruginosa* except *E. coli*. Addition of 1% PEG to the extract resulted in reduction of antibacterial activity. The percentage reduction of 90% for *P. thonningii* was achieved against all four bacteria.

The highest percentage reduction of 95% was achieved for *B. maughamii* and *D. rotundifolia* against *B. cereus* where the MIC values increased from 0.5 to 10.0 mg ml^{-1}. The lowest percentage reduction of 20.0% was recorded for *H. amoenus* against *P. aeruginosa* where the MIC increased from 4.0 to 5.0 mg ml^{-1}. However, *B. maughamii* was found not to have tannins.

Tetracycline hydrochloride (antibiotic) was used to determine the antibacterial activity of four tests organisms that is *B. cereus*, *E. coli*, *E. faecalis* and *P. aeruginosa*. The antibacterial activity of tetracycline was obtained at 100 μg ml^{-1} against Gram-positive and Gram-negative bacteria. All crude extracts showed lower activity against Gram-positive and Gram-negative bacteria as compared to the antibiotic.

5.2.2 Serial dilution method

The serial dilution method was used to determine the MIC values of the plant extracts. This method was used in order to confirm the results obtained from agar-dilution method (discussed above), since it was reported as the most reliable method than the others. In general, the method is quick and works well with bacteria (Eloff 1998b).

The acetone extract of *D. rotundifolia* showed antibacterial activity against *B. cereus* with an MIC of 0.39 mg ml^{-1}. Antibacterial activity was previously detected in ethanol and water extracts of leaf material from *D. rotundifolia*, and showed antibacterial activity against *Bacillus subtilis* with MIC value of 0.40 mg ml^{-1} (McGaw 2000). However, some researchers disagree with the results as they found no antibacterial activity in the ethanol and water extracts (Reid *et al*. 2001). Previous studies reported that ethyl acetate extract of leaf material of *Dombeya burgessisae* showed antibacterial activity against *B. subtilis* with an MIC value of 12.5 mg ml^{-1} while the ethanol extract of leaf material of *Dombeya cymosa* showed antibacterial activity against *B. subtilis* with MIC value of 1.56 mg ml^{-1} (Reid *et al*. 2005). Some researchers concluded that the solvent used to extract plant material had an effect on antibacterial activity. In the current study acetone was used and low MIC values were obtained as compared to the previous studies. In the present study, the extract of *D. rotundifolia* showed antibacterial activity against *E. coli* with an MIC value of 0.78 mg ml^{-1} while some researchers have found no activity (McGaw 2000). The ethanol extract of leaf material from *D. cymosa* was previously screened for antibacterial activity. The inhibitory activity was obtained against *E. coli* with an MIC value of 1.56 mg ml^{-1}. However, no activity was obtained in the ethanol extract of *D. burgessisae* (Reid *et al*. 2005). It was also reported that no differences in the activity of the ethyl acetate extracts from *D. burgissisae* and *D. cymosa* against *E. coli* with an MIC value of 12.5 mg ml^{-1}. In general, the MIC values that were obtained as compared to other studies showed that acetone is the best solvent used for extracting antibacterial activity. This finding is supported by Eloff (1998a) who used acetone for extracting plant material and found that greater antibacterial activity were obtained than in the case of methanol, ethanol and water.

The acetone extract of leaf material of *P. thonningii* showed antibacterial activity against *B. cereus* with an MIC value of 0.39 mg ml^{-1} as compared to Akinpelu and Obuotor (2000) who used 60% methanol as extractant solvent and the activity that was obtained against *B. subtilis* (same family as *B. cereus*) yielded an MIC value of 0.31 mg ml^{-1}. In the current study, the extract of *P. thonningii* showed antibacterial activity against *E. coli* with an MIC value of 0.78 mg ml^{-1}. Some researchers used the stem bark of *P. thonningii* and found that the activity (MIC value of 10.0 mg ml^{-1}) was obtained against *E. coli* (Akinpelu and Obuotor 2000).

The extracts of *P. thonningii* in 100% acetone had higher antibacterial activity against *P. aeruginosa* (Table 4.7). The results indicate that the acetone crude extracts of all plant species exhibit antibacterial activities towards the Gram-positive bacteria. The acetone extracts in some plant samples were more active against Gram-positive than Gram-negative bacteria, for example, *B. cereus* (Gram-positive bacteria) whereas *E. coli*, *E. faecalis* and *P. aeruginosa* (Gram-negative bacteria). These results were supported by Eloff (2000) who considered 100% acetone because it extracted more antibacterial activity in Combretaceae.

Most noticeably, no tannins were recorded in the extract of *B. maughamii* and the results obtained showed that antibacterial activity is not due to tannins. However, other authors reported that some secondary metabolites are responsible for antibacterial activity. For example: alkaloids (Masuda *et al*. 1991, Benevides *et al*. 1999, Dorman and Deans 2000), flavonoids (Havsteen 1983) and terpenes (Aharoni *et al*. 2005).

The extract of *B. salicina* yielded no noticeable differences in activity towards *B. cereus*, *E. faecalis* and *P. aeruginosa*.

In this study, greater antibacterial activity was observed against *E. faecalis* and *P. aeruginosa*. Some researchers reported ethanol extracts for *D. cymosa* with an MIC value

of 0.195 mg ml^{-1} to have a greater activity (Reid *et al*. 2005). Other researchers reported antibacterial activity on the leaves of *D*. *rotundifolia* and also discovered that Gram-negative bacteria were more resistant (McGaw 2000; Reid *et al*. 2001). Previous studies showed that the extracts of medicinal plants were more active against the Gram-positive than Gram-negative bacteria (Vlietinck *et al*. 1995; Rabe and Van Staden 1997), because of their thick murein layer which prevents the entry of inhibitors.

In this study, PEG was used to determine the effect of tannins on antibacterial activity and the MIC values obtained in this combination was higher as compared to the extracts which contained no PEG. In addition, there was a reduction of antibacterial activity in all plant samples. The extract of *B*. *maughamii* and *H*. *amoenus* showed the same activity against *B*. *cereus* and *P*. *aeruginosa* after addition of 1% PEG to the plant material and this could imply that the activity is not due to tannins (Table 4.7), in particular for *B*. *maughamii*. The increase in MIC value of *H*. *amoenus* could be as a result of tannin binding since tannins are recorded in this plant. These results suggest that PEG may bind to plant secondary metabolites other than tannins.

Addition of 1% PEG to plant extracts resulted in the reduction of antibacterial activity. The highest percentage reduction of 97.8% was achieved for *B*. *maughamii* against *E*. *faecalis*, where the MIC values increased from 0.195 to 3.13 mg ml^{-1}. The lowest percentage reduction was 50% for *B*. *maughamii* against *P*. *aeruginosa*, *B*. *salicina* against *E*. *coli* and *P*. *thonningii* against *E*. *coli*. Most noticeable the percentage reduction of all plant extracts varies for different bacteria, suggesting that the bacteria are sensitive to different bioactive compounds in the extract.

The antibacterial activity of the five plants extracts containing 1% PEG was weak compared to that of the extract without PEG (control). The results show that tannins may be responsible for antibacterial activity and this could suggest that there may be other secondary metabolites with antibacterial activity. In contrast, Karou *et al*. (2005) reported

74

that polyphenols from extracts of *Combretum micranthum*, *Khaya senegalensis*, *Pterocarpus erinaceus* and *Sida acuta* may be responsible for antibacterial activity while Basri and Fan (2005), Irobi *et al*. (1994) and Scalbert (1991) reported that tannins contributed to the antibacterial activity of *Quercus infectoria*. Tannins or polyphenolic compounds are known to have *in vitro* antimicrobial activity, but have limited *in vivo* application due to low bioavailability and strong binding properties to proteins (Eloff *et al*. 2005). Even though tannins are known to have good antimicrobial activity, paradoxically some organisms, for example, *Candida sp*, are capable of using tannins as a source of carbon (Scalbert 1991; Irobi *et al*. 1994).

Acetone as a solvent alone was tested and no inhibition of bacteria was observed against Gram-negative and Gram-positive bacteria. When 1% PEG was added to the acetone, weak antibacterial activity was obtained against Gram-positive and Gram-negative bacteria (MIC > 12.5 mg ml^{-1}). This suggested that PEG is not toxic to the bacteria and inhibition of bacterial growth was observed at MIC value of 12.5 mg ml^{-1}. Similar results were obtained by Dow chemicals (1974) and Lockard *et al*. (1979) who observed low toxicity of PEG. Also, Olitzky (1965) and Chen (2005) reported the antimicrobial properties of propylene glycol and found that Gram-positive and Gram-negative bacteria were resistant. Therefore, PEG increased the survival of the bacteria. Previous studies have reported that PEG solutions may have a potential value in medicine as a topical antibacterial activity (Chirife *et al*. 1983). Thus PEG has been used in the preparation of hydrophilic ointment bases incorporating a wide variety of therapeutic materials such antibacterial, antibiotics and steroids. Polyethylene glycol can also be used in foods as a coating, blinder, or plasticizing or body agent (Furia 1975).

Antibacterial activity of tetracycline was demonstrated against Gram-positive (MIC 0.01 mg ml^{-1}) Gram-negative bacteria (MIC 0.02 and 0.03 mg ml^{-1}). The five plant extracts showed weak antibacterial activity against Gram-positive and Gram-negative bacteria as compared to tetracycline. Our results obtained with tetracycline are different from other

investigators who reported tetracycline resistance (83.3%) in the synergism assay against some bacteria (Nascimento *et al*. 2000).

5.3 Thin layer chromatography and bioautography

The active components of plant extracts were analysed by TLC on silica gel. This method is rapid and an effective means of obtaining an analytical fingerprint of plant extracts (McGaw *et al*. 2002). In the current study, thin layer chromatography plates were run in duplicate and one set was used as the reference chromatogram, the other set was used for bioautography. The results showed that there were bioactive compounds in different fractions (hexane, ethyl acetate and butanol). The most bioactive compounds were obtained in the ethyl acetate fractions. Some researchers have used TLC chromatograms and discovered some differences in chemical composition between the different plant parts of different trees (Zschocke *et al.* 2000; Eloff 2001; Kotzé and Eloff 2002). The leaf extracts of *B. maughamii* demonstrated more antibacterial activity as compared to other plant extracts (in the ethyl acetate fraction). The bioautography method worked well (more antibacterial activity observed) with *Pseudomonas aeruginosa*, in contrast low activity were observed with *B. cereus*, *E. coli* and *E. faecalis*. *Pseudomonas aeruginosa* cause serious infections in human and there are no conventional antibiotics against this bacteria (CDC NNIS system 1999; Madigan *et al*. 2000).

Antimicrobial components (extract of *B. salicina*) were present in the crude methanol, butanol and ethyl acetate fractions. The bioautography confirmed the antibacterial activity of *P. thonningii* in both, ethyl acetate and butanol fractions, low antibacterial compounds were found in crude methanol and ethyl acetate fractions. The extract of *H. amoenus* was more active in ethyl acetate and butanol fractions. The big difference in the antibacterial activity and vanillin-visualised chromatograms may indicate that the antibacterial compounds are present in low concentrations or that they do not react with the vanillin spray reagent (Eloff *et al*. 2005).

CHAPTER 6

CONCLUSIONS

6. CONCLUSIONS

❖ Higher tannin levels were recorded in all plant samples extracted with 100% acetone than with 70% acetone.

❖ PEG and PVPP (both at 0.5%) removed less tannin in 100% acetone extracts as compared to 1% PEG and PVPP with PEG being more effective than PVPP.

❖ In serial dilution method, the extract of *B. maughamii* and *P. thonningii* showed higher antibacterial activity against *E. faecalis* and *P. aeruginosa* with an MIC value of 0.195 mg ml^{-1} and this suggests that the active compounds found in the extract could be of pharmaceutical importance.

❖ The minimum inhibitory concentration (MIC) obtained in this study showed that five plant extracts have antibacterial activity and they have potential for the treatment of various diseases of livestock. No cyto-toxicity test was conducted in this study.

❖ The results obtained in this study showed that the activity observed in plant samples could be due to tannins and other secondary metabolites that bind PEG.

❖ The addition of 1% PEG to plant extracts resulted in the highest percentage reduction of antibacterial activity, up to 97.8% for serial dilution method compared with 95% recorded in agar-dilution method. The serial dilution method is preferred for *E. faecalis* whereas the agar-dilution method is highest for *B. cereus* bacteria, respectively

❖ The thin layer chromatograms of all plant extracts showed antibacterial compounds in the crude methanol, hexane, butanol and ethyl acetate but not aqueous fractions.

CHAPTER 7

RECOMMENDATIONS

7. RECOMMENDATIONS

- ❖ Further studies should be conducted to identify the types of tannins found in the four plant extracts and whether PEG binds to soluble or insoluble tannins.

- ❖ Larger scale extraction methods and fractionations of extracts should be carried out in order to further investigate the extracts with low MIC values.

CHAPTER 8
REFERENCES

REFERENCES

Afolayan AJ, Meyer JJM (1997) The antimicrobial activity of 3, 5, 7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57: 177-181

Aharoni A, Jongsma MA, Bouwmeester HJ (2005) Volatile science? Metabolic engineering of terpenoids in plants. Trends in plant science 10(12): 594-602

Ahmed F, Selim MST, Shilpi JA (2005) Antibacterial activity of *Ludwigia adscendens*. Fitoterapia 76: 473-475

Akinpelu DA, Obuotor EM (2000) Antibacterial activity of *Piliostigma thonningii* stem bark. Fitoterapia 71(4): 442-443

Anderson RA, Sowers JA (1968) Optimum condition for binding of plant phenols to insoluble polyvinyl pyrrolidone. Phytochemistry 7: 293-301

Arnold HJ, Guluman M (1984) Pharmacopoeia of traditional medicine in Venda. Journal of Ethnopharmacology 12: 35-74

Atawodi SE, Usman M, Bulus ST, Atawodi JC, Wakawa L, Ameh (2000) Herbal treatment of some protozoal and parasitic diseases of poultry in the middle belt of Nigeria. In: Paper presented at the International Workshop on Ethnoveterinary Practices. Arewa House, Kaduna, Nigeria

Barahona R, Lascano CE, Cochran R, Morrill J, Titgemeyer EC (1997) Intake, digestion, and nitrogen utilization by sheep fed tropical legumes with contrasting tannin concentration and astringency. Journal of Animal Science 75: 1633-1640

Basri DF, Fan SH (2005) The potential of aqueous and acetone extracts of galls of *Quercus infectoria* as antibacterial agents. Indian Journal of Pharmacology 37(1): 26-29

Bate-Smith EC (1975) Phytochemistry of Proanthocyanidins. Phytochemistry 14: 1107-1113

Begue WJ, Kline RM (1972) The use of tetrazolium salts in bioautographic procedures. Journal of Chromatography 64: 182-184

Benevides PJC, Sartorelli P, Kato MJ (1999) Phenylpropanoids and neolignans from *Piper regnellii*. Phytochemistry 52: 339-343

Bizima N (1994) Traditional veterinary practice in Africa. Schriftreiner GTZ, No 243, Eschborn, Germany

Bryant JP, Reichardt BP, Clausen TP (1992) Chemically mediated interactions between woody plants and browsing mammals. Journal of Range Management 45: 18-24

Bruneton J (1999) Pharmacognosy phytochemistry medicinal plants (2nd edn). Lavoisier, Paris

CDC NNIS system (1999) National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1990-May 1999. American Journal of Infection Control 27: 520-532

Chirife J, Herszage L, Joseph A, Bozzini JP, Leardini N, Kohn ES (1983) *In vitro* antibacterial activity of concentrated Polyethylene glycol 400 solutions. Antimicrobial Agents and Chemotherapy 24(3): 409-412

Chung KT, Wong TY, Wei CI, Huang YW, Lin Y (1998a) Tannins and human health: A review. Critical Reviews in Food Science and Nutrition 38: 421-464

Coates-Palgrave K, Coates-Palgrave M, Coates-Palgrave P (1985) Everyone's Guided to Trees in South Africa. National Book Printers, Cape Town

Cole MD (1992) The significance of the terpenoids in the Labitae. In: Harley RM, Reynolds T (ed). Advances in Labitae Science. Royal Botanic Gardens, Kew, pp 315-324

Conserve Africa Foundation (2004) Medicinal Plants and Natural Products http://www.conserveafrica.org.uk/nat_products.html

Cornell (2000) Tannins: Chemical analysis http://www.ansci.cornell.edu/plants/toxicagents/tannin/chem_anl.html

Cornell (2001) Medicinal plants for livestock beneficial or toxic? http://www.ansci.cornell.edu/plants/medicinal/index.html

Cork SJ, Krockenberger AK (1991) Methods and pitfalls of extracting condensed tannins and other phenolics from plants: Insights from investigations on *Eucalyptus* leaves. Journal of Chemical Ecology 17(1): 123-134

Cowan MM (1999) Plant products as antimicrobial agents. Clinical Microbiology Review 12: 564-582

Croteau, R B (1998) "The Discovery of Terpenes." In Discoveries in Plant Biology Vol. I, eds. Shain-Dow Kung and Shang-Fa Yang. Singapore: World Scientific

Cunningham AB (1993) African Medicinal Plants: setting priorities at the interface between conservation and primary healthcare

Cushnie TPT, Lamb AJ (2005) Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 26(5): 343-356

Dalziel JM (1937) The Useful Plants of West Tropical Africa. Crown Agents for the Colonies London. pp 135-137

Dauskardt R (1990) The changing geography of traditional medicine: urban herbalism on the Witwatersrand, South Africa. Geographical Journal 22: 275-283

Deng C, Rong G, Tian H, Tang Z, Chen X, Jing X (2005) Synthesis and characterization of poly (ethylene glycol)-*b*-poly ($_L$-lactide)-*b*-poly ($_L$-glutamic acid) triblock copolymer. Polymer 46: 653-659

De Moor P. P, Pooley E, Neville G, Barichievy J (1977) The vegetation of Ndumu Game Reserve, Natal: A quantitative physiognomic survey. Annals of the Natal Museum 23: 239-272

Doke CM, Vilakazi BW (1972) Zulu-English Dictionary. 2nd ed. Witwatersrand University Press, Johannesburg

Dold AP, Cocks ML (2001) Traditional veterinary medicine in the Alice district of the Eastern Cape Province. South African Journal of Science 97: 375-379

Doner LW, Bécard G, Irwin P L (1993) Binding of flavonoids by polyvinylpolypyrroli-done. Journal of Agricultural and Food Chemistry 41: 753-757

Dorman HJ, Deans SG (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88: 308-316

Dow Chemical U.S.A (1974) Polyethylene glycols handbook. Dow Chemical Midland, Co., Mich

Duri ZJ, Scovill JP, Huggins JW (1994) Activity of a methanolic extract of Zimbabwean *Crinum macowanii* against exotic RNA viruses *in vitro*. Phytotherapy Research 8: 121-122

Eloff JN (1998a) Which extractant should be used for the screening and isolation of Antimicrobial components from plants? Journal of Ethnopharmacology 80: 1-8

Eloff JN (1998b) A sensitive and quick microplate method to determine the MIC of Plant extracts for bacteria. Planta Media 64: 711-713

Eloff JN (2000) On expressing the antibacterial activity of plant extracts-a small first step in applying scientific knowledge to rural primary health care. South African Journal of Science 96: 116-118

Eloff JN (2001) Antibacterial activity of Marula (*Sclerocarya birrea* A. rich.) Hochst. Subsp. *Caffra* (Sond.) Kokwaro) (Anarcardiaceae) bark and leaves. Journal of Ethnopharmacology 76: 305-308

Eloff JN, Famakin JO, Katerere DRP (2005) *Combretum woodii* (Combretaceae) leaf extracts have high activity against Gram-positive and Gram-negative bacteria. African Journal of Biotechnology 4(10): 1161-1166

FAO/ IAEA (2000) Quantification of tannins in tree foliage. Working Document, IAEA, Vienna, pp1-26

Fennell CW, Van Staden J (2001) *Crinum* species in traditional and modern medicine. Journal of Ethnopharmacology 78: 15-26

Ford JE, Hewitt D (1979) Protein quality in cereals and pulses. 3. Bioassays with rats and chickens on sorghum (*Sorghum vulgare* Pers.), barley and field beans (*Vicia faba* L.). Influence of polyethylene glycol on digestibility on the protein in high-tannin grain. British Journal of Nutrition 42: 325-340

Furia TE (1975) Handbook of food additives, 2nd ed. CRC Press, Cleveland, Ohio

Furness HD, Breen C. M (1980) The vegetation of seasonally flooded areas of the Pongolo River Floodplain. Bothalia 13: 217-231

Gesler WM (1984) Health Care in Developing Countries. Resource Publications in Geography. Association of American Geographers, Washington D.C, USA, pp 88

Ghosal S, Saini KS, Razdan S (1985) *Crinum* alkaloids. Their chemistry and biology. Phytochemistry 24(10): 2141–2156

Ghosal S, Singh SK, Unnikrshnan SG (1990) Effects of stress on alkaloid metabolism in *Crinum asiaticum*. Phytochemistry 29 (3): 805–811

Gleadow RM, Woodrow IE (2002) Constraints on effectiveness of cyanogenic glycosides in herbivore defense. Journal of Chemical Ecology 28(7): 1301-1313

Grange JM, Davey RW (1990) Antibacterial properties of propolis (blue glue). Journal of Royal Society of Medicine 83

Grierson DS, Afolayan AJ (1999) Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. Journal of Ethnopharmacology 66(1): 103-106

Guri-Fakim A, Subratty AH (1996) Validating ethno-medicines with a view to
developing new drugs

http://www.irfd.org/events/wfsids/virtual/papers/sids_guribfakim3.pdf

Hagerman AE (1987) Radial diffusion method for determining tannins in plant extracts.
Journal of Chemical Ecology 13: 437-449

Hagerman AE, Robbins CT (1987) Implications of soluble tannin-protein complexes
for tannin analysis and plant defense mechanisms. Journal of Chemical Ecology 13:
1243-1259

Hagerma AE (1988) Extraction of tannin from fresh and preserved leaves. Journal of
Chemical Ecology 14(2): 452-461

Hagerman AE, Butler LG (1994) Assay of condensed tannins or flavonoid oligomers
and related flavonoids in plants. Methods in Enzymology 234: 429-437

Harborne JB (1984) Phytochemical methods. Chapman and Hall Ltd Great Britain
Richard Clay. pp 7, 84

Harborne AE, Baxter H (1993) Phytochemical Dictionary: A handbook of bioactive
compounds from plants. Taylor and Francis, London

Harborne JB, Baxter H (1999) The handbook of natural flavonoids. Vol 1& 2. John
Wiley & Sons, Chichester

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992.
Phytochemistry 55 (6): 481-504

Haslam E (1989) Plant Polyphenols-Vegetable Tannins Revisited. In: Chemistry and Pharmacology of Natural Products. Cambridge University Press, Cambridge

Haslam E (1996) Natural polyphenols (vegetable tannins) as drugs: possible modes of action. Journal of Natural Products 59: 205-215

Hatano T, Yazaki K, Okonogi A, Okuda T (1991) Chemical and Pharmaceutical bulletin 39: 1689

Havsteen B (1983) Flavonoids, a class of natural products of high pharmacological potency. Biochemical Pharmacology 32: 1141-1148

Henry GAF, Thompson K, Moss CJ, Edwards E, Thorpe PC (1994) Seed persistence: A correlation between seed longevity in the soil and ortho-dihydroxyphenol concentration. Functional Ecology 8(5): 658-664

Hilliard OM (1983) In: Flora of Southern Africa (Asteraceae). Vol. 33. Asteraceae. Lo.eistner, O.A. ed. Botanical Institute of South Africa. pp 61-310

Hoareau L, DaSilva EJ (1999) Medicinal plants: A re-emerging health aid. Journal of Biotechnology 2(2).
http://www.ejbiotechnology.info/content/vol2/issue2/full/2/

Holetz FB, Pessini GL, Sanches NR, Cortez AG, Nakamura CV, Filho BPD (2002) Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases 97(7): 1027-1031

Hufford CD, Funderburk JM, Morgan JM, Roberts LW (1975) Two antimicrobial alkaloids from heartwood of *Liriodendron tulipifera* L. Journal of pharmaceuticals Sciences 64: 789-792

Hutchings A, Scott AH, Lewis G, Cunningman AB (1996) Zulu Medicinal Plants:
An inventory. University of Natal Press; Pietermaritzburg, South Africa. pp 213-215

Ibrahim MA, Nwude N, Ogunsusi RA, Aliu YO (1984) Screening of West African
plants for anthelmintic activity. ILCA Bulletin No. 17. International Livestock Centre
for Africa, Addis Ababa, Ethiopia, pp 19-23

Ibrahim R (2006) Commercialization of Advanced Bio-reactor.
http://www.symbiosisonline.com/aug05_bioreactor.htm

Immelman WFE, Wicht CL, Ackerman DD (1973) Our Green Heritage: The South
African Book of Trees. Tafelberg-Uitgewers Beperk, Cape Town

Irobi ON, Moo-Young M, Anderson WA, Daramola SO (1994) Antimicrobial activity
of bark extracts of *Bridelia ferruginea* (Euphorbiaceae). Journal of
Ethnopharmacology 43(3): 185-190

Irvine FR (1961) Woody plants of Ghana. London: Oxford University Press. pp 216-
217

Jones DE (1965) Banana tannin and its reaction with polyethylene glycol. Nature 206:
299-300

Jones WT, Mangan JL (1977) Complexes of the condensed tannins of sainfoin
(*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary
mucoprotein, and their reversal by polyethylene glycol and pH. Journal of the Science
of food and Agriculture 28: 126-136

Jones DA (1998) Why are so many food plants cyanogenic? Phytochemistry 47: 155-162

Jones PR, Anderson MD, Nielsen JS, Høj PB, Møller BL (2000) The biosynthesis, degradation, transport and possible function of cyanogenic glucosides. In JT Romeo, R Ibrahim, L Varin, V DeLuca, eds, Evolution of Metabolic Pathways. Recent Advances in Phytochemistry, Vol 34. Pergamon, Amsterdam, pp 191-247

Kelmanson JE, Jäger AK, Van Staden J (2000) Zulu medicinal plants with antibacterial activity. Journal of Ethnopharmacology 69: 241-246

Kemper KJ (1999) Longwood Herbal Task Force
http://www.mcp.edu/herbal/default.htm

Kostova I (2001) *Fraxinus ornus* L. Fitoterapia 72: 471-480

Kotzé M, Eloff JN (2002) Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). South African Journal of Botany 68: 62-67

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. American Journal of Medicine 113 suppl 9B: 71S-88S

Kumar R, Vaithiyanathan S (1990) Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Animal Feed Science and Technology 30: 21-38

Liengme CA (1981) Plants used by the Tsonga people of Gazankulu. Bothalia 13: 501-518

Lockard JS, Levy RH, Congdom WC, DuCharme LL (1979) Efficacy and toxicity of the solvent polyethylene glycol 400 in monkey model. Epilepsia 20: 77-84

Mabogo D E N (1990) The Ethnobotany of the Vhavenda. Unpublished Master of Science Thesis, University of Pretoria

Madigan MT, Martinko JM, Parker J (2000) Brock Biology of microorganisms. Prentice Hall International, Inc., New Jersey

Makkar HPS, Becker K, Abel H, Szegletti C (1995) Degradation of condensed tannins by rumen microbes exposed to Quebracho tannins (QT) in rumen simulation technique (RUSITEC) and effects of QT on fermentative processes in the RUSITEC. Journal of the Science of food and Agriculture 69: 495-500

Makkar HPS (2003) Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Research 49: 241-256

Marcus S (1992) A preliminary study on the Anthelmintic properties of *Terminalia glaucescens* in cattle in the Northwest Province of Cameroon. An ethnoveterinarian approach. DVM thesis. Utrecht: University of Utercht, Faculty of Veterinary Medicine. pp 2

Marin PD, Grayer RJ, Veitch NC, Kite GC, Harborne JB (2001) Acacetin glycosides as taxonomic markers in *Calamintha* and *Micromeria*. Phytochemistry 58: 943-947

Martin GJ (1995) Ethnobotany: A methods Manual. Chapman Hall, London, pp 7

Martin M, Mathias E, McCorkle CM (2001) Ethnoveterinary medicine: An annotated bibliography of community animal healthcare, Essex, England

Masika PJ, Van Averbeke W, Sonandi A (2000) Use of herbal remedies by small-scale farmers to treat livestock diseases in central Eastern Cape Province, South Africa. Journal of South African Veterinary Association 71: 87-91

Masuda T, Inazumi A, Yamada Y, Padolina WG, Kikuzaki H, Nakatani N (1991) Anti-
microbial phenyl propanoids from *Piper sarmentosum*. Phytochemistry 30: 3227-
3228

Matekaire T, Bwakura TM (2004) Ethnoveterinary medicine: a potential alternative to
Orthodox animal health delivery in Zimbabwe. International Journal of Applied
Research in Veterinary Medicine 2(4): 269- 273

Mathias E (2001) Introducing ethbnoveterinary medicine. http://www.ethnoweb.com

Mathias E (2004) Review Article Ethnoveterinary medicine: harnessing its potential
Veterinary Bulletin 74(8), 27N – 37N

McCorkle CM (1986) An introduction to ethnoveterinary research and development.
Journal of Ethnobiology 6:129-149

McCorkle CM, Mathias-Mundy E, Van Schillhorn-van-Veen TW (1996)
Ethnoveterinary Research and Development. IT Studies and Indigenous Knowledge
and Development. Intermediate Technology Publications, London. Medicine in
Africa. Africa 62: 29-93

McDougall GJ, Morrison IM, Stewart D, Hillman JR (1996) Journal of the Science of
Food and Agriculture 70: 133-150

McGaw LJ, Jäger AK, Van Staden J (2000) Antibacterial, Anthelmintic and Anti-
amoebic activity in South African Medicinal Plants. Journal of Ethnopharmacology
72: 247-263

McGaw LJ, Jäger AK, Van Staden J (2002) Variation in antibacterial activity of
Schotia species. South African Journal of Botany 68: 41-46

McNabb WC, Waghorn GC, Peters JS, Barrr TN (1996) The effect of condensed tannins in *Lotus pedunculatus* on the solubilization and degradation of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39; rubisco) protein in the rumen and the sites of rubisco digestion. Baroda Journal of Nutrition 76: 535-549

Meagher LP, Lane G, Sivakumaran S, Tavendale MH, Fraser K (2004) Characterization of condensed tannins from Lotus species by thiolytic degradation and electrospray mass spectrometry. Animal Feed Science and Technology 117(1-2): 151-163

Mesfin T, Obsa T (1994) Ethiopian traditional veterinary practices and their possible contribution to animal production and management. In Early methods of animal disease control. Review of Science and Technology 13(2): 417-424

Meyer JJM, Afolayan AJ (1995) Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 47:109-111

Middleton E Jr, Chithan K (1993) The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In: JB Harborne, Editor. The flavonoids: Advances in research since 1986, Chapman and Hall, London, UK

Minja MMJ (2002) Status of indigenous knowledge integration in the development process in Tanzania with particular focus on the validation of ethnoveterinary practices: lessons and experiences[1]. Paper presented at the Regional workshop (on Indigenous Knowledge, in Kampala, 4-5 December

Mirutse G (2001) An ethnobotanical study of medicinal plants used by the Zay people in Ethiopia, CBM: Skiftserie 3: 81-99

Mitscher LA, Leu R-P, Bathala MS, Wu W-N, Beal JL (1972) Antimicrobial Agents from higher plants. I. Introduction, Rationale, and Methodology. Journal of Biological Sciences 35(2): 157-166

Moll E. J (1978) The vegetation of Maputaland. Trees of South Africa 29: 31-59

Moll E J, White F (1978) The Indian Ocean Coastal Belt. In: Werger, M. J.A (ed). Biogeography and ecology of Southern Africa, Dr. W. Junk, The Hague. pp 561-598

Mongrand S, Badoc A, Patouille B, Lacomblez C, Chavent M, Bessoule J (2005) Chemotaxonomy of the Rubiaceae family based on leaf fatty acid composition. Phytochemistry 66(5): 549-559

Mulu A, Tessema B, Derbie F (2004) In vitro assessment of the antimicrobial potential of honey on common human pathogens. Ethiopian Journal of Health Development 18(2): 107-111

Nascimento GGF, Locatelli J, Freitas PC, Silva GL (2000) Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Brazilian Journal of Microbiology 31: 247-256

Nuñez-Hernández G, Wallace JD, Holechek JL, Galyean ML, Cárdenas M (1991) Condensed tannins and nutrient utilization by lambs and goats fed low-quality diets. Journal of Animal Science 69: 1167-1177

Okuda T, Hatano T, Yazaki K (1991) Chemical and Pharmaceutical bulletin 31: 333

Olitzky I (1965) Antimicrobial properties of a propylene glycol based topical therapeutic agent. Journal of Pharmaceutical and Sciences 54: 787-788

O'Neill MJ, Lewis JA (1993) The renaissance of plant research in the pharmaceutical
industry. In: Kinghorn AD, Balandrin MF (eds) Human medicinal agents from plants.
ACS Symposium Series 534. American Chemical Society, Washington, USA, pp 48-55

Palmer E, Pitman N (1961) Trees of South Africa. A.A. Balkema, Amsterdam.

Paz EA, Cerdeiras MP, Fernandez J, Ferreira F, Moyna P, Soubes M, Vázquez A, Vero
S, Zunino L (1995) Screening of Uruguayan medicinal plants for antimicrobial
activity. Journal of Ethnopharmacology 45: 67-70

Penso G (1980) The role of WHO in the selection and characterization of medicinal
plants (vegetable drugs). Journal of Ethnopharmacology 2: 183-188

Pooley E (1993) The complete Field Guide to Trees of Natal, Zululand and Transkei.
Natal Flora Publication Trust, Natal herbarium, Durban

Prance GT (1994) Introduction. In Chadwick DJ, Marsh J (eds.) Ethnobotany and the
search for new drugs. Wiley, Chichester (Ciba Foundation Symposium 185)
pp 1-3

Pretorius SJ, Joubert PH, Evans AC (1988) A re-evaluation of the molluscicidal
properties of the torchwood tree, *Balanites maughamii* . South African Journal of
Science 84: 201-202

Price ML, Stromberg AM, Butler LG (1979) Tannin content as a function of grain
maturity and drying conditions in several varieties of Sorghum bicolor (L.) Moench.
Journal of Agricultural Food Chemistry 27: 1270-1274

Rabe T, van Staden J (1997) Antibacterial activity of South African plants used for
medicinal purposes. Journal of Ethnopharmacology 56: 81-87

Rabe T, Mullholland D, Van Staden J (2002) Isolation and identification of antibacterial compounds from *Vernonia colorata* leaves. Journal of Ethnopharmacology 80: 91-94

Rajan S, Sethuraman M (1997) Traditional veterinary practices in rural areas of Dindigul district, Tamilnadu, India. Indigenous Knowledge and Development Monitor 5: 7-9

Rajnikant, Dinesh, Kamini (2005) Weak C-H…O hydrogen bonds in alkaloids: an overview. Bulletin of Materials Science 28 (3): 187–198

Rasoanaivo P, Ratsimamanga-Urveg S (1993) Biological evaluation of plants with reference to Malagasy flora. Monogram for the IFS-Napreca Workshop on Bioassays. Antananarivo, Madagascar. pp 9-43, 72-83

Razafimandimbison SG, Moog J, Lantz H, Maschwitz U, Bremer B (2005) Re-assessment of monophyly, evolution of myrmecophytism, and rapid radiation in Neonauclea s.s (Rubiaceae). Molecular Phylogenetics and Evolution 34(2): 334-354

Reed JD (1985) Gravimetric determination of soluble phenolics including tannins from leaves by precipitation with trivalent ytterbium. Journal of Food Science and Agriculture 36: 255-261

Reid JD (1995) Nutritional toxicology of tannins and related polyphenols in forage legumes. Journal of Animal Science 73: 1516-1528

Reid KA, Jäger AK, Van Staden J (2001) Pharmacological and phytochemical properties of *Dombeya rotundifolia*. South African Journal of Botany 67: 349-353

Reid KA, Jäger AK, Light ME, Mulholland DA, Van Staden J (2005) Phytochemical screening of *Sterculiaceae* species and isolation of antibacterial compounds. Journal of Ethnopharmacology 97(2): 285-291

Rios JL, Simeón S, Jiménez FJ, Zafra-Polo MC, Villar A (1985) Reagents for screening medicinal plants by thin-layer chromatography: a review. Fitoterapia 58: 153-162

Robbins CT, Hanley TA, Hagerman AE, Hjeljord O, Baker DL, Schwartz CC, Mautz WW (1987) Role of tannins in defending Plants against ruminants: Reduction in protein availability. Ecology 68(1): 98-107

Rogers CB (1998) Cycloartenoid dieno acids and lactones from *Combretum erythrophyllum*. Phytochemistry 47: 2069-2076

Saijo R, Nonaka G-I, Nishioka I (1989) Chemical and Pharmaceutical bulletin 37: 2063

Salminem JP (2003) Effects of sample drying and storage, and choice of extraction solvent and analysis method on the yield of birch leaf hydrolysable tannins. Journal of Chemical Ecology 29(6): 1289-1305

SAS (1997) SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina, USA

Selmar D (1999) Biosynthesis of cyanogenic glucosides, glucosinolates and nonprotein amino acids. In M Wink, ed, Biochemistry of Plant secondary metabolism, Annual Plant Reviews Vol 2, Sheffield Academic Press, CRC Press, pp 79-150

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30(12): 3875-3883

Schofield P, Mbugua DM, Pell AN (2001) Analysis of condensed tannins: a review. Animal Feed Science and Technology 91: 21-40

Silanikove N, Perevolotsky A, Provenza FD (2001) Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants. Animal Feed Science and Technology 91: 69-81

Snyder LR, Kirkland JJ (1979) Introduction to modern liquid chromatography. John Wiley, New York, USA.

Swaleh A (1999) Ethnoveterinary medicine in Ormaland-Kenya. MSc Thesis. University of Edinburgh, UK

Taiz L, Zeiger T (2002) Plant physiology. 3rd ed. Sinauer Associates, Inc., Publishers.Sunderland, Massachusetts. pp 287

Thomas V, Grant R (1998) Sappi tree spotting. Jacaranda Education Ltd, Johannesburg

Toda M, Okubo S, Ohnishi R, Shimamura T (1989) Antibacterial and bactericidal activities of Japanese green tea. Japan Journal of Bacteriology 45: 561-566

Van der Merwe D, Swan GE, Botha CJ (2001) Use of ethnoveterinary medicinal plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province of South Africa. Journal of South African Veterinary Association 72(4): 189-196

Van Wyk B, Van Wyk P (1997) Field Guide to Trees of Southern Africa. Struik Publisher, Cape Town

Van Wyk B, Van Outshoorn B, Gericke N (1997) Medicinal plants of South Africa, Briza Publications Pretoria, South Africa. pp 304

Vasa (2006) The Vasa's battle Polyethylene glycol (PEG) treatment htt://www.fos.su.se/~magnuss/peg.html

Vlietinck AJ, Van Hoof L, Totté J, Lasure A, Vanden Berghe D, Rwangabo PC, Mvukiyumwami J (1995) Screening of a hundred Rwandese medicinal plants for anti-microbial and antiviral properties. Journal of Ethnopharmacology 46: 31-47

Von Breitenbach F (1986) National list of Indigenous Trees. Dendrological foundation, Pretoria

Von den Driesch, A(1989). Geschichte der Tiermedizin [History of veterinary medicine]. Verlag Georg DW Callway GmbH & Co, München, Germany.

Wagner H, Bladt S (1995) Plant Drug Analysis. A thin layer chromatography atlas. 2nd Ed. Springler-Verlag, New York, USA. pp 384

Waller GR, Nowacki EK (1978) Alkaloid Biology and metabolism in plants, Plenum Press, New York. pp 293

Waterman PG, Mole S (1994) Analysis of phenolic plant metabolites. Blackwell Scientific Publications, Oxford

Watt JM, Breyer-Brandwick MG (1962) Medicinal and Poisonous Plants of Southern and Eastern Africa. E & S. Livingstone LTD, Edinburg, UK. 2nd ed. pp 1457

Wilson CG, Thomas NW (1984) Interaction of tissues with polyethylene glycol vehicles. International Journal of Pharmaceuticals 5: 94-97

World Health Organization (2006) Traditional Medicine
http://www.who.int/medicines/areas/traditional/en/index.html

Yff BTS, Lindsey KL, Taylor MB, Erasmus DG, Jager AK (2002) The pharmacological screening of *Pentanisia prunelloides* and the isolation of the antibacterial compound palmitic acid. Journal of Ethnopharmacology 79: 101-107

Yu F, Moughan PJ, Barry TN, McNabb WC (1996) The effect of condensed tannins from heated and unheated cotton seed on the ileal digestibility of amino acids for the growing rat and pig. British Journal of Nutrition 76: 359-371

Zschocke S, Rabe T, Taylor JLS, Jäger AK, Van Staden J (2000) Plant part substitution- a way to conserve endangered medicinal plants? Journal of Ethnopharmacology 71: 281-299

APPENDICES

Appendix A

Table A1 Partial analysis variance of tannins in 70% acetone extracts with and without PEG

Source	DF	Mean Square	F value	Pr> F
Species	4	27.569	2866.87	0.0001
Rep	3	0.0029	0.30	0.8238
PEG	2	21.526	2238.51	0.0001
Species* PEG	8	4.0119	417.20	0.0001

R-Square 0.98

Table A2 Partial analysis variance of tannins in 70% acetone extracts with and without PVPP

Source	DF	Mean Square	F value	Pr> F
Species	4	42.527	1596.55	0.0001
Rep	3	0.0166	0.62	0.6013
PVPP	2	10.479	393.41	0.0001
Species* PVPP	8	1.6284	61.13	0.0001

R-Square 0.97

Appendix B

Table B1 Partial analysis variance of tannins in 100% acetone extracts with and without PEG

Source	DF	Mean Square	F value	Pr> F
Species	4	30.612	9991.54	0.0001
Rep	3	0.0082	2.67	0.0482
PEG	2	86.268	28157.7	0.0001
Species* PEG	8	9.158	2989.16	0.0001

R-Square 0.99

Table B2 Partial analysis variance of tannins in 100% acetone extracts with and without PVPP

Source	DF	Mean Square	F value	Pr> F
Species	4	56.000	243037.7	0.0001
Rep	3	0.0068	2.93	0.0343
PVPP	2	38.507	16528.9	0.0001
Species* PVPP	8	4.3224	1855.34	0.0001

R-Square 0.99

Table B3 Partial analysis variance of 100 % acetone extracts (controls)

Source	DF	Mean Square	F value	Pr> F
Species	4	40.4016	777.63	0.0001
Rep	3	0.033	0.63	0.5981

R-Square 0.97