A survey of plants used to treat livestock diseases in the Mnisi community, Mpumalanga, South Africa, and investigation of their antimicrobial activity

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Declaration

I declare that this thesis titled “A survey of plants used to treat livestock diseases in the Mnisi community, Mpumalanga, South Africa, and investigation of their antimicrobial activity” hereby submitted to the University of Pretoria has not been submitted by me or any other person for a degree at this at any other institution of higher learning, it is my own original work and all material contained herein has been duly acknowledged.

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Mr E.T. KHUNOANA

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List of Abbreviations

ATCC	American Type Culture Collection
BIA	Biofilm Inhibitory Activity
CFU	Colony-Forming Units
DNA	Deoxyribonucleic Acid
DOS	Doctrine of Signatures
EVM	Ethnoveterinary Medicine
FMD	Foot and Mouth Disease
GLTFCA	Greater Limpopo Trans-Frontier Conservation Area
GLTP	Greater Limpopo Trans-Frontier Park
IC_{50}	Inhibitory concentration
INT	*p*-Iodonitrotetrazolium
MH	Müller-Hinton
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
NGO	Non-Governmental Organization
RRA	Rapid Rural Appraisal approach
SD	Sabouraud Dextrose
SI	Selective Index
TB	Tuberculosis

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Abstract

Livestock owners in developing countries with limited access to expensive orthodox veterinary services have been using ethnoveterinary practices for animal healthcare for generations. The knowledge is passed on from generation to generation orally, but there is a concern that as time goes on the information may be lost or inadequate information may be passed on to future generations, thus there is an urgent need to document the available knowledge of ethnoveterinary medicine.

The aim of this study was to document and investigate plant species that are used as ethnoveterinary medicine by the Mnisi community at Bushbuckridge in Mpumalanga Province. Following the Rapid Rural Appraisal (RRA) guidelines, a survey was conducted to document the plant species that are used as ethnoveterinary medicine within the Mnisi community. It was found that older males from the age of 40 were more knowledgeable than females and young people. Plant remedies were more frequently used than pharmaceutical drugs, with cattle being the dominantly treated animals. Eleven plant species belonging to seven families were reported by the farmers, and fresh plants from the wild were commonly used to prepare the remedies. The remedies were prepared as decoctions, infusions, pastes and extracted sap.

Frequently used plants were selected and tested for their antibacterial (against planktonic and biofilm forms), antifungal, antimycobacterial and cytotoxic activity. Traditional extraction (decoctions and infusions prepared with tap water) and organic solvent extraction (extracts prepared in the laboratory using distilled water and acetone) were used to prepare the extracts which were tested against *Escherichia coli, Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*, *Mycobacterium tuberculosis* and *Mycobacterium bovis.* Out of all the tested samples the acetone extracts had better antibacterial activity with MIC values ranging from as low as 0.09 to 0.27 mg/ml compared to the samples extracted using traditional methods (decoctions and infusion). However, water extracts had the highest antifungal activity with MIC values ranging from 0.02 to 2.50 mg/ml compared to organic solvent extracts which had MIC values ranging from 0.63 to 2.50 mg/ml. Water extracts also had the highest antimycobacterial activity compared to acetone, with MIC values ranging from 0.02 to 1.2 mg/ml. *Elephantorrhiza obliqua* acetone extract had the best antibacterial activity with

an MIC value of 0.09 mg/ml against *P. aeruginosa*, while *E. obliqua* water extracts had the highest antifungal activity with MIC values of 0.02 to 0.04 mg/ml against *Aspergillus fumigatus*. *Schotia brachypetala* acetone extracts inhibited *Enterococcus faecalis* biofilm by 113% and 135% at zero and 24 hours of bacterial growth respectively, while *E. obliqua* acetone extracts had values of 64% and 83% at these time periods, indicating that they were good inhibitors of biofilm formation and also had the capacity to act against mature biofilms.

Seven out of nine tested plant extracts (78%) were non-toxic to moderately cytotoxic while only two plant extracts were relatively toxic against Vero cells. *Aloe marlothii* (infusion) and *Schotia brachypetala* (aqueous extract) were non-toxic with the highest IC_{50} values of 0.205 mg/ml and 0.105 mg/ml respectively.The selectivity index was used to relate the cytotoxicity to the antimicrobial activity of the plant extracts. Most of the plant extracts had values below 10 against all the tested microbes, implying that the biological activity of the plant extracts may be associated with toxicity. However it must be noted that toxicity *in vitro* might not translate to toxicity *in vivo,* thus *in vivo* studies must be carried out qualify the toxicity.

In summary, plants were used in the Mnisi community for the treatment of common diseases in livestock. Only a few plant species were used, but these plant species used by the Mnisi community were related in some cases to those used in nearby areas such as the Vhembe region (Luseba and Tshisikhawe, 2013) and the Greater Giyani district (Luseba and Van der Merwe, 2006). Traditionally prepared remedies were generally more active against fungi and mycobacteria and less active against bacteria. They were also less toxic than the organic solvent extracts. However, *in vivo* studies are also necessary to support the traditional use of the remedies against diseases in livestock in terms of validating the efficacy but also assessing their potential toxicity.

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Chapter 1

Introduction

The use of traditional medicine dates back from ancient times and it remains part of the heritage of indigenous people. Ancient societies used traditional medicine to treat both human and animal diseases (Luseba and Van der Merwe, 2006). Farmers in the 21st century have continued to use traditional practices to sustain their livestock health, production and also to prevent and control diseases such as retained placenta, diarrhoea, gallsickness, fractures, eye inflammation, general unwellness, fertility problems, gastrointestinal ailments, heartwater, helminthosis, coughing, redwater and reduction of ticks. The application of these traditional practices constitutes ethnoveterinary medicine (EVM), which is used mostly in developing countries like South Africa where there are many rural areas consisting of small scale farmers that have limited access to Western veterinary services or expensive pharmaceutical drugs, thus EVM serves as a low-cost and readily available alternative (McGaw and Eloff, 2008).

South Africa is home to a wide range of temperate flora comprising an estimated 24 000 plant species (Germishuizen and Meyer, 2003). This region is also home to a rich diversity of cultures and traditions, which is reflected by the use of plants as medicine. It is estimated that over 72% of people in the country use plants as medicine (Keirungi and Fabricius, 2005) for both human and animal health care. Many rural Africans continue to have limited access to Western veterinary services and may prefer to use traditional medicines to treat both humans and animals. Traditional knowledge is commonly passed on from one generation to the next in oral format (McGaw and Eloff, 2008). According to McGaw and Eloff (2008) small-scale farmers of different ethnic groups, including Tswana, Tsonga, Xhosa and Zulu, use traditional remedies to treat animal diseases owing to its low cost, convenience and ease of administration. In most ethnic groups, this ancient knowledge was not recorded or made public to people outside the immediate culture, and this is one of the reasons why different ethnic groups use different traditional medicines to treat the same diseases in this region (Ramadwa, 2010). Previous studies (Luseba and Van der Merwe, 2006; Maphosa and Masika, 2010) report that farmers from different ethnic groups use different medicinal plants to treat the same diseases. Additional reasons for this are that different ethnic groups are situated in different parts of the country where availability of different plants varies, thus a particular ethnic group may have access to certain plants while others do not.

Modern pharmaceuticals are gradually failing to prevent or cure infectious diseases as a result of different microorganisms adapting to the exposure of antimicrobial agents by

developing resistance to them. Some antimicrobial agents that are known to be associated with development of resistance include gentamicin, methicillin, vancomycin and carbenicillin (Van Vuuren and Holl, 2017). In order for antimicrobial resistance to develop, there must be contact between the microbial strain and the antibiotic; resistance will then transferred by DNA to daughter organisms or directly to other members of the same species (Khachatourians, 1998). The agents lose their efficacy when they are inappropriately ingested by patients or when they are used extensively in agriculture as growth catalysts and to prevent infections. The misuse of these agents results in the development of resistant microbial strains such as methicillin-resistant *Staphylococcus aureus,* multi and extremely drug-resistant *Mycobacterium tuberculosis*, carbenicillin-resistant *Pseudomonas aeruginosa*, β-lactamase-resistant *Escherichia coli*, chloramphenicol-resistant *Candida albicans* (Van Vuuren and Holl, 2017). However plants may be useful as alternative antimicrobial agents not only to resistant strains but also to normal strains, for example *Spirostachys africana* Sond. extracts had MIC values of 39.06 µg/ml against β-lactamase resistant *E. coli.* Similar results were reported for *Knowltonia vesicatoria* (L.f.) Sims against *M. tuberculosis* (Van Vuuren and Holl, 2017). In a study done by Fabricant and Farnsworth (2001) it was found that 122 compounds of defined structure used globally in the drug industry were obtained from only 94 species of plants. Newman and Cragg (2012) also reported 175 compounds from natural products as sources of new drugs over a recent 30 year period (1981-2010). Almost 80% of the drugs had an ethnomedical use identical or related to the current use of the active elements of the plant. Thus it is important to investigate plants to find new unrelated compounds to counteract the development of resistance to current compounds.

EVM serves as an alternative or as a complementary medication for curing or preventing bacterial, fungal and helminthic diseases, as well as other maladies such as ticks and tick-borne diseases. It is important to investigate the plants growing in different ecosystems and regional climates as they produce a variety of secondary metabolites that have different functions such as defence against herbivores, parasites and diseases (Cowan, 1999). The chemicals obtained from plants consist of complex chemical structures that are not available in synthetic compound libraries. It is estimated that there are about 250 000 plant species throughout the world and only 5-15% of these species have been tested for bioactive compounds (Pieters and Vlietinck, 2005). In South Africa, relatively few surveys have been conducted and even fewer ethnoveterinary remedies have been tested for efficacy and safety (McGaw and Eloff, 2008).

Traditional medicinal knowledge is passed on from generation to generation orally, and there is a concern that as time goes on the information may be lost or inadequate information may be passed on to future generations. Thus there is an urgent need to document the available

knowledge since most ethnic groups are increasingly willing to share knowledge of their EVM practices. Ethnoveterinary surveys are considered the most effective way of obtain information about the methods of preparing and administration of traditional medicine to animals (Luseba and Van der Merwe, 2006). Thus in this study a survey was conducted following the Rapid Rural Appraisal approach (RRA) to document EVM practices. Farmers, herdsman and animal technicians were interviewed in various dipping tanks within the Mnisi community, Bushbuckridge in the Mpumalanga province of South Africa and 11 plant species belonging to seven families were reported to be used. Plant material was collected with the guidance of the respondents and identified. Three plants which were frequently used for prevalent diseases (diarrhoea and black quarter) and those less investigated in literature were selected for evaluation and plant samples were then prepared and subjected to laboratory analysis to evaluate the biological activities of the plants used in EVM. The biological activities evaluated included antibacterial, antimycobacterial and antifungal activity and cytotoxicity of the plant samples.

1.1 Aims of the study

The aim of this study was to document and investigate plant species that are used as ethnoveterinary medicine by the Mnisi community at Bushbuckridge in Mpumalanga and to evaluate their antimicrobial activity and cytotoxicity.

1.2 Objectives

The objectives of this study were to:

- Conduct a survey to obtain information on plants used in EVM, their methods of preparation and administration
- Collect plants, identify their scientific names and prepare voucher specimens
- Select plants from the survey that are used to treat microbial-related ailments
- Perform laboratory assays to test and compare the activity of the plants prepared in the traditional way and using organic solvents against selected bacterial, fungal and mycobacterial pathogens
- Test the plant extracts for cytotoxicity

Chapter 2

Literature review

2.1 History of EVM

Historical records reveal that people were already practicing the treatment of animals using traditional methods over 14 000 years ago. Initially the dog was treated, then by 9 000 BC treatment of sheep and goats followed in the fertile Nile Valley, then cattle followed in Egypt and Anatolia from 4 000 BC and 6 000 BC respectively (Wanzala *et al*., 2005). This evolution continued with treatment of pigs and horses in Sri Lanka at around 3 000 BC as they were considered to have a good association with man, with the method of treatment used being Ayurvedic medicine (the Indian healing system). The relationships between man and animals were based on economic, cultural, social and religious beliefs attached to each type of animal, and it was during this time that veterinary medicine evolved specifically to take care of the health of domesticated animals. Religious and medical roles were combined for treatment, for example in the veterinary Papyrus of Kahun (1900 BC) there are records of veterinary therapeutic techniques of Egyptian healers (priest-healers) (Wanzala *et al*., 2005).

2.2 Traditional diagnosis of diseases affecting livestock in different regions of South Africa

Veterinary ailments affecting livestock have a high impact throughout the world, resulting in losses in livestock production. The most frequently recorded ailments in South Africa are diarrhoea, gallsickness, fractures, eye inflammation, general unwellness, female cattle reproductive issues, gastrointestinal ailments, heartwater, helminthosis, coughing and redwater as well as ticks and tick-borne diseases (Luseba and Van der Merwe, 2006; Van der Merwe *et al*., 2001). According to a survey done amongst Tswana-speaking people of Madikwe in the North-West province, gallsickness and diarrhoea in their livestock is caused by the consumption of green grass as it is believed to have a high incidence during the growth season (Luseba and Van der Merwe, 2006). Additionally, diarrhoea and loss of appetite are associated with the presence of internal parasites although the parasites could not be differentiated if they were tapeworms or flukes (Luseba and Van der Merwe, 2006). Heartwater is believed to be caused by ticks (*Amblyomma haebraeum*) and is suspected when an over-excited animal runs wild and falls dead with froth from the mouth (Luseba and

Van der Merwe, 2006). Black quarter is suspected when an animal fails to wake up after previously limping and showing swelling in the legs. Newcastle disease is linked with greenish diarrhoea (Luseba and Van der Merwe, 2006). In another survey done amongst Tsonga-speaking people, farmers also believe that diarrhoea is associated with internal parasites, and heartwater is caused by excessive accumulation of blood in the head that results from tick bites. The farmers also believe that drinking dirty water results in redwater disease (Van der Merwe *et al.*, 2001). Also, in a survey done amongst a Venda-speaking population, diarrhoea, worm infestation, tick bites and eye infections were prevalent (Luseba and Tshisikhawe, 2013). Tick-borne diseases included redwater and heartwater diseases. Worm infestations were highly linked to diarrhoea and lack of appetence (Luseba and Tshisikhawe, 2013). In other cases, plants used as treatment are linked with the symptomatic observations of the disease, for example the reddish colour of the underground parts of *Rhoicissus tridentata* (L.f.) Wild & R.B.Drumm (Vitaceae) and *Drimia sanguinea* (Schinz) Jessop (Asparagaceae) are linked with the reddish urine of infected animals (Van der Merwe *et al*, 2001). This concept is known as the doctrine of signatures (DOS) which refers to the belief that that plant signatures such as colour, shape and common name can be used to treat diseases with similar properties, or signatures (Bennett, 2007). DOS is still used, for example in Europe the plant *Sanguinaria canadensis* L. (Papaveraceae) (bloodroot) is used to treat blood related ailments, and the plant contains an orange-red alkaloid known as sanguinarine (Bennett, 2007). In Israel, plants with yellow sap or yellow flowers are used to treat hepatitis, for example *Rhamnus alaternus* L. (Rhamnaceae) (Dafni and Lev, 2002). Similarly in Nigeria, the bark and root of *Entandrophragma utile* (Dawe & Sprague) Sprague (Meliaceae) is used as a blood tonic. Plant shape can also be used as a signature, for example *Aconitum napellus* L. (Ranunculaceae) which is shaped like an eye is used to treat ocular problems, and *Fittonia albivenis* (Lindl. ex Veitch) Brummitt (Acanthaceae) which is liver shaped is used to treat liver pains (Dafni and Lev, 2002). Similarly, shape can sometimes be associated with the animal that causes a particular disease, for example the inflorescence *Pallenis spinosa* (L.) Cass (Compositae) resembles a spider, and therefore it is used to treat spider bites in Israel (Dafni and Lev, 2002).

2.3 Plants used as ethnoveterinary medicine in South Africa

Farmers in rural areas often prefer to use traditional remedies because of their greater availability, easy accessibility, low costs and apparent effectiveness (Luseba and Tshisikhawe, 2013). Farmers believe that plants are safe to use and that they do not have any side effects on their livestock. In addition, farmers also believe that there is no need for

withdrawal periods before the consumption of meat from animals treated with herbal remedies (Luseba and Tshisikhawe, 2013). South African researchers have engaged with various communities in different regions within the country to document ethnoveterinary use of plants. The Rapid Rural Appraisal (RRA) approach has been used where surveys were done to obtain information about ethnoveterinary medicine from various ethnic groups (McGaw and Eloff, 2008). A review done by McGaw and Eloff (2008) comprised a compilation of the complete systematic ethnoveterinary record of South African plants that were reported from 1938 to 2008. About 200 plant species from over 80 families were reported to be used to treat common livestock diseases by various ethnic groups. The review included plants which are used amongst Tsonga-speaking people of the Greater Giyani municipality, where 19 plant species belonging to 12 plant families were reported and the most common families were the Euphorbiaceae and Fabaceae with 4 plants each (Luseba and Van der Merwe, 2006). The review also included plants which are used by the Tswana-speaking people of Madikwe in the North West province, where 45 plant species from 24 plant families used to treat cattle were documented. Plants such as *Acacia tortilis* (Forssk.) Hayne (Leguminosae), *Sclerocarya birrea* (A.Rich.) Hochst (Anacardiaceae), *Senna italica* Mill. (Leguminosae), *Searsia lancea* (L.f.) F.A.Barkley (Anacardiaceae), *Peltophorum africanum* Sond. (Leguminosae), *Elephantorrhiza elephantina* (Burch.) Skeels (Leguminosae), *Ziziphus mucronata* Willd. (Rhamnaceae) and *Ziziphus zeyheriana* Sond. (Rhamnaceae) were reported to be commonly used, possibly due to their high levels of tannin-protein complexes. Tannins are naturally occurring polyphenolic chemical compounds with high molecular weight (500-3000 Da), the tannins attach to proteins resulting in protein complexes which are associated with astringent, antimicrobial and anthelmintic effects on animals. These compounds are found in leaves, wood and bark (Hassanpour *et al*., 2011; Van der Merwe *et al*., 2001).

Relatively few surveys specific to ethnoveterinary knowledge were discovered in publications from 2008-2018 in South Africa. Out of all surveys from 2008-2018, 83% were in the Eastern Cape Province and 17% in Venda, Limpopo province. Only two provinces (Eastern Cape and Limpopo) out of nine were investigated for ethnoveterinary practices for the past ten years. From all the identified plant species, only a small number have been investigated for their biological activity. Therefore, more research needs to be done in the ethnoveterinary field and more complete investigation of ethnoveterinary practices amongst different ethnic groups around South Africa needs to be done document our ethnoveterinary knowledge more completely (McGaw and Eloff, 2008).

From the few ethnoveterinary surveys conducted amongst different communities of various ethnic groups, it was found that the Venda people of the Vhembe region in Limpopo

province used a total of 49 plant species but only 38 plant species belonging to 19 plant families and 37 genera were identified and documented (Luseba and Tshisikhawe, 2013). The Fabaceae had the highest number of plant species reported with six species followed by the Asteraceae and Rubiaceae (three species each), and then the Combretaceae and Euphorbiaceae (two species each) (Luseba and Tshisikhawe, 2013). The Fabaceae was also reported to be the dominantly used family by the Tswana-speaking people of Madikwe (Luseba and Van der Merwe, 2006).

In another survey done by Kambizi (2016) it was found that the communities of Pondoland (Xhosa-speaking) also use indigenous plant species for ethnoveterinary purposes. The survey revealed that 23 plants from 18 families were used to treat livestock. Farmers of the Amatola Basin (Xhosa-speaking) in the Eastern Cape indicated that wounds and myiasis were serious health problems in cattle and resulted in a decrease in animal production, causing severe economic losses (Soyelu and Masika, 2009).

Intestinal parasites are a worldwide problem, causing heavy production losses in animals. They are commonly found in developing countries, mainly because of poor management practices and inadequate control measures in association with warm temperatures. It was discovered that in most ethnoveterinary surveys, medicinal plants are commonly used for the control of these parasites (Maphosa and Masika, 2010). In a survey done by Maphosa and Masika (2010) it was found that 28 plant species from 20 families were used as ethnoveterinary medicine to treat gastrointestinal parasites in goats. The survey was done in the Eastern Cape and 30 individuals were interviewed. The same plant species were also identified in a similar survey including both intestinal and external parasites done by Sanhokwe *et al.* (2016) in Kwezi and Ntambethemba villages in the Eastern Cape Province. In another survey done by Mwale and Masika (2009), most of the poultry farmers interviewed used more than nine plant species to treat gastrointestinal parasites in their chickens, with *Aloe ferox* being the most popular medicinal plant used for controlling these parasites. Again, *Aloe ferox* was the most commonly used plant along with *Prunus persica* (L.) Batsch (Rosaceae) and *Phytolacca heptandra* Retz. (Phytolaccaceae) in a survey done by Soyelu and Masika (2009). *Aloe ferox* Mill. (Xanthorrhoeaceae) was also reported to be used to treat ticks and mites by the farmers in Kwezi and Ntambethemba villages in the Eastern Cape (Sanhokwe *et al*, 2016). One of the greatest problems with verminosis is the huge resistance of many nematodes to the current available anthelmintic groups. In some farms, *Haemonchus contortus* is resistant to every anthelmintic on the market, making sheep production in these areas impossible (Sanhokwe *et al*, 2016). Thus EVM can be used to find new unrelated compounds, or mixtures of plant components in

active extracts or fractions that act synergistically against nematodes, reducing the likelihood of resistance developing so quickly.

There is a relationship between plants used for animal health care and plants used for human health care. For example, *Hippobromus pauciflorus* Radlk. (Sapindaceae) is used to treat diarrhoea in humans (Bisi-Johnson *et al* 2010) and it is also used to wash wounds in animals (Soyelu and Masika, 2009). The Vhembe community use *Turraea obtusifolia* Hochst. (Meliaceae) to treat wounds in animals by directly applying it on the wound (Luseba and Tshisikhawe, 2013) while it is also used by the people of Pondoland to treat diarrhoea (Madikizela *et al.*, 2012). Some plants like *Z. mucronata* and *Bulbine abyssinica* A.Rich. (Asphodelaceae) are used for similar ailments such as diarrhoea and gastro-intestinal parasites (McGaw and Eloff, 2008; Maphosa and Masika, 2010). In other surveys the same plant species were used for different ailments. According to Maphosa and Masika (2010) *Gunnera perpensa* L. (Gunneraceae) is used by farmers of the Eastern Cape to treat intestinal parasites in goats while in the study by Gerstner (1941) the same plant is used in both animals and woman for expulsion of afterbirth. Luseba and Tshisikhawe (2013) stated that the farmers of the Vhembe region in the Limpopo province use *Ximenia americana* L. (Olacaceae) to treat wounds and calving difficulties while Van der Merwe *et al.* (2001) stated that the same plant was used to treat intestinal parasites. Hutchings *et al*. (1996) also reported that *Ptaeroxylon obliquum* (Thunb.) Radlk. (Rutaceae) is used as an anthrax remedy and against ticks in cattle while Maphosa and Masika (2010) state that it is also used for intestinal parasites in goats. *E. elephantina* is used by the Pedi people of the Limpopo province to treatment pneumonia and tick-borne diseases in cows while it is also used to treat chest pains, heart conditions and stomach ailments in humans (Kudumela and Masoko, 2018). Some of the plants that belong to the same genus are used to treat similar ailments. *E elephantina* and *Elephantorrhiza burkei* Benth. (Leguminosae) roots are used to treat sexually transmitted infections, as a blood purifier, to treat eye infections and also to treat infections in cattle (Mathabe *et al.*, 2006).

2.4 Challenges of traditional remedies

South Africa is home to 10% of the world's total number of higher plant species but only a small portion of the plants have been scientifically validated for biological activity (Ramadwa, 2010). However, even though there is a lack of activity in a laboratory-based screening system it does not necessarily mean EVM are not effective (McGaw and Eloff, 2008). Plants are known to produce phytochemicals for protection against microbial attack and animal herbivory and these phytochemicals are also able to protect humans from diseases

associated with microorganisms. For example tannins are used against diarrhoea as anti-inflammatory, haemostatic pharmaceuticals and as antiseptics (Motlhatlego *et al*., 2018). Proanthocyanidins which are condensed tannins also have antioxidant, freee radicle scavaging, anticarcinogenic and anti-inflammatory properties (Motlhatlego *et al*., 2018).

Limitations to medicinal use of plants are that their growth is seasonal, thus sometimes when they are needed they are not available, and also over-harvesting can result in extinction of certain plants as some species are not able to recover quickly after such harvests, particularly when bark or underground parts are used. Inadequate storing of the plants after harvest may also lead to contamination, and misidentification of the plants is also a problem as some plant species look similar. The dosage that is administered to animals by EVM practitioners is also not precise, thus solutions to these disadvantages need to be obtained through direct interaction with the users of the medications (Ramadwa, 2010; Luseba and Van der Merwe, 2006; Van der Merwe *et al.*, 2001). Some plants used as EVM are dangerous and have toxic effects if consumed in high amounts, for example in a study done amongst Tswana people, it was found that at least nine plant species including *Boophone disticha* (L.f.) Herb. (Amaryllidaceae)*, Croton gratissimus* Burch. (Euphorbiaceae)*, Phyllanthus* spp*., Ricinus communis* L*.* (Euphorbiaceae)*, Spirostachys africana* Sond. (Euphorbiaceae)*, Solanum incanum* L. (Solanaceae)*, Solanum panduriforme* E. Mey. (Solanaceae)*, U. sanguinea* and *Withania somnifera* (L.) Dunal (Solanaceae) had toxic effects on the animals (Van der Merwe *et al.*, 2001).

2.5 Plant toxicity

The investigation of toxicity in plant extracts is very important in order to determine the potential toxic effects towards the animals being treated. There are about 600 known toxic plant species in South Africa, but the side effects and toxicity associated with use of EVM plants are rarely reported (McGaw and Eloff, 2010). This may be due to the relatively small quantities used by EVM practitioners, although there are plants that are used as EVM that are toxic, for example *B. disticha, R. communis* and *Solanum* species. Thus, evaluation of the toxic effects of EVM remedies is important. The methods of preparation as well as application and dosages are critical factors to account for when evaluating a traditional remedy. According to Van der Merwe *et al*. (2001) it is believed that the small amounts of plant material used in medicines are the reason for the scarcity of reported toxic effects. McGaw and Eloff (2008) recommended that further research be done to optimize dosing and most effective concentrations of EVM remedies.

2.6 Plants as antimicrobial agents

Antibiotics have been used since the 1940s to treat patients with infectious bacterial diseases. These pharmaceuticals performed very well and reduced illness and death rates from conditions such as pneumonia, meningitis and tuberculosis (Van Vuuren and Holl, 2017; Khan *et al.*, 2015). However, some antibiotics have been used inadequately for so long that some virulent bacteria have gained resistance to them, making the treatments less effective (Khan *et al*, 2015; Kaikabo, 2009). Inadequate consumption of antimicrobials results when patients do not complete the course of the prescribed antimicrobial, take lower doses or take the antimicrobials for irrelevant diseases, and as a result resistant microbial agents can still survive and continue to grow (Khan *et al*., 2015). Antibiotic resistance occurs when an antimicrobial agent loses its ability to kill bacteria. The organisms will then continue to grow and cause infection, even in the presence of the antibiotic (Khan *et al*., 2015; Kaikabo *et al*., 2009; Khachatourians, 1998). Some bacteria are naturally resistant to certain types of antibiotics, but most become resistant through a natural genetic mutation or by acquiring resistance genes from other bacteria which then leads to resistance to different types of antibiotics over time (Van Vuuren and Holl, 2017; Khan *et al*, 2015; Kaikabo, 2009).

The rapid increase in antibiotic resistance is now a global problem and one of the major scientific issues to date. The development of new antibiotics is slow and difficult work but bacterial resistance is decreasing our arsenal of existing drugs, posing a catastrophic threat as ordinary infections become untreatable. Preventative action is needed to help reduce resistance and this can be achieved by encouraging the development of new drugs from alternative sources. Thus, traditional medicine may serve as an alternative for curing or preventing bacterial, fungal, tick-borne and other diseases, as well as parasite infestations. Hence it is important to investigate plants growing in the different ecosystems of South Africa as they produce a variety of secondary metabolites that have different functions such as defence against herbivores, parasites and diseases. The chemicals obtained from the plants consist of complex chemical structures that are not available in synthetic compound libraries. With an estimated 250 000 plant species worldwide and only up to 15% of species having been investigated for their biological properties (Pieters and Vlietinck, 2005), there is much opportunity for discovery of interesting phytochemicals.

Billions of people in developing countries have been using medicinal plants and other natural products to treat infectious diseases (Van Vuuren and Holl, 2017). The high demand of these products has resulted in an ever growing market, and South Africa is no exception to this. This may be due to the diversity of southern African plant species and the rich cultural

heritage of traditional healing practises (Van Vuuren and Holl, 2017). The study of medicinal plants has been fruitful as it has yielded novel and active antimicrobial compounds. These compounds have aided in the production of antimicrobial pharmacological drugs. The therapeutic properties exerted by the plants are believed to be due to secondary metabolites (for example alkaloids, saponins, steroids, terpenols, tannins, glycosides, resins, flavonoids, volatile oils) found in the plants (Van Vuuren and Holl, 2017; Cowan, 1999). These secondary metabolites have been found *in vitro* to have antimicrobial properties (Cowan, 1999). The aim of using plants as a source of therapeutics is to isolate bioactive compounds for direct use as drugs (morphine, digoxin and vinblastine), to also use the compounds to synthesise bioactive compounds with higher activity and/or lower toxicity (metformin, oxycodone and nabilone). Therefore there is a need to investigate the possible presence of novel and active antimicrobial compounds from medicinal plants (Van Vuuren and Holl, 2017).

Plants such as *A. tortilis, S. italica, P. africanum, E. elephantina, Z. mucronata* and *Z. zeyheriana* were reported to be highly used by Tswana people for animal health care due to their high levels of tannins (Van der Merwe *et al.*, 2001). The pharmacological effect of tannins include antibacterial, antifungal, antioxidant, astringent and mucoprotectant effects, thus tannin containing plants were used to treat diarrhoea, burns and blood cleansing (Van der Merwe *et al.*, 2001). *Aloe greatheadii* Schönland (Xanthorrhoeaceae) and *Aloe zebrina* Baker (Xanthorrhoeaceae*)* leaves contain slimy sap which has a cooling and soothing effect on inflamed skin lesions, thus it is used to treat burn wounds and also to protect the wounds from contamination (Van der Merwe *et al.*, 2001). Since ethnoveterinary practitioners mostly use water as a solvent, research has also turned to using water as an extraction solvent when performing laboratory *in vitro* assays to try to replicate the same results or activity obtained by the ethnoveterinary practitioners.

Most research has been done *in vitro* on antimicrobial properties of EVM plants. Excellent minimum inhibitory concentration (MIC) values were observed for crude acetone extracts of the following plant species: *Maesa lanceolata* Forssk. (Primulaceae) (MIC = 0.02 mg/ml), *Bolusanthus speciosus* (Bolus) Harms (Leguminosae*), Hypericum roeperianum* Schimp. ex A.Rich. (Hypericaceae) and *Morus mesozygia* Stapf (Moraceae) (MIC = 0.04 mg/ml) (Elisha *et al*., 2016). These extracts were tested against against the vaccine strain of *Bacillus anthracis* and they showed promising antibacterial activity and it was suggested that *M. lanceolata* and *H. roeperianum* extracts could be used as disinfectants (Elisha *et al*., 2016).

Chapter 3

A survey of plants used to treat livestock diseases in the Mnisi community, Mpumalanga, South Africa

3.1 Introduction

Ethnoveterinary medicine (EVM) comprises a complex system of beliefs, knowledge, skills and practice relating to animal health care. The field includes traditional veterinary theory, animal husbandry, diagnostic procedures and surgical methods (Van der Merwe *et al*., 2001; McCorkle, 1986). Livestock owners have been using EVM for many years to prevent and control diseases. EVM practices vary by ethnicity and locality. Different ethnic groups use different plant species based on the knowledge that was provided by their elders, ancestors or other community members of the same ethnic group. Previous studies (Luseba and Van der Merwe, 2006; Maphosa and Masika, 2010) reported that farmers from different ethnic groups use different medicinal plants to treat the same diseases. This is also because different ethnic groups are situated in different parts of the country where the availability of certain plants varies, thus each ethnic group has access to certain plants while some may not have access to these plants.

EVM practices are locality and culture-specific and generalization of results from one area to another is not possible. This is because different areas can use the same plants for treating different diseases or they can use different plants for controlling the same diseases. The biological activity of a plant might differ from one region to another due to environmental factors such as climate (rainfall and temperature) and the geographical position (topography and soil) (Dubey *et al*., 2004). These factors affect the production of the metabolites which are responsible for various biological activities of the plants, hence the generalization of the results between areas may not be useful as the activity of the plant depends on the favourable conditions within the plant's surrounding (Dubey *et al*., 2004).

Traditional medicine used for both animal and human health care plays a vital role in the development of new pharmaceuticals. Traditional medicine used for human treatment developed parallel with that for animal treatment, yet human treatment has more documentation compared to animal treatment. The migration of indigenous people to urban areas has resulted in the loss of indigenous traditions in favour of Western-derived traditions

(Maphosa and Masika, 2010; McGaw and Eloff, 2008; Luseba and Van der Merwe, 2006; Van der Merwe *et al*., 2001). There is an urgent need to document the knowledge about the traditional use of plants in EVM before it is extinct since the knowledge is passed from one generation to the next orally (Maphosa and Masika, 2010; McGaw and Eloff, 2008; Luseba and Van der Merwe, 2006; Van der Merwe *et al.*, 2001).

Livestock animals which are in close proximity to wildlife (game) are at risk of contracting various diseases which may be passed from wild animals to livestock. Wild animals are reservoirs of diseases that can be dangerous to livestock health (De Bruin, 2015). These diseases can decrease the productivity of livestock and even cause death. Livestock animals are infected when they come into contact with wildlife, for example African buffaloes in the Kruger National Park are the maintenance hosts of Foot and Mouth Disease (FMD) which affects all cloven-hoofed animals such as cattle, pigs, sheep and goats (De Bruin, 2015). Corridor Disease affects cattle that graze on the same pastures where African buffaloes are grazing or have recently been grazing. Thus the present study was carried out in an area with wildlife-livestock interface in order to contribute to an understanding of the close interaction between wildlife, livestock and humans.

The Mnisi community is located within the savannah ecosystem in the north-eastern corner of the Bushbuckridge Local Municipality in Mpumalanga province, South Africa. The study area was selected due to its complex biodiversity as there is a close interaction between wildlife, livestock and humans, which may result in contraction of different diseases. The area consists mainly of a Shangaan-speaking community of over 40 000 people. The selection of this area was based on its classification as one of South Africa's 14 rural poverty nodes and an existing community upliftment programme undertaken by the University of Pretoria in the area. Approximately 75% of the Mnisi community boundary is at the wildlife–livestock interface between communal livestock areas and various provincial and private game reserves, most of which are part of the Greater Limpopo Trans-Frontier Conservation Area (GLTFCA). Geographically the Mnisi community is located adjacent to the GLTFCA, which is made up of the Greater Limpopo Trans-Frontier Park (GLTP), which is a combination of South Africa's Kruger National Park, Zimbabwe's Gonarezhou National Park and the Limpopo National Park in Mozambique. The Mnisi community's proximity to areas inhabited by wildlife puts the community and their livestock at risk of contracting infectious diseases (De Bruin., 2015).

The survey was conducted following methods described by Weckerle *et al*. (2018) and Heinrich *et al*. (2017) which correlates with the Rapid Rural Appraisal (RRA) approach, which is used for quickly obtaining a preliminary understanding of a situation where specific

research techniques are chosen from a wide range of options and where there is an assumption that all the relevant parts of a local system cannot be known in advance (Beebe, 1995). The local system is best understood by combining the expertise of a multidisciplinary team that includes locals, while combining information collected in advance, direct observations and semi-structured interviews, and time should be structured to ensure team interaction as part of an iterative process (Beebe, 1995). The RRA consists of three basic concepts, namely a system perspective, triangulation of data collection, and iterative data collection and analysis (Beebe, 1995). These three concepts provide a conceptual foundation for rapid appraisal and a rationale for the selection of specific research techniques. The RRA should be based on what the participants in the system believe to be the critical elements, their relative importance, and how they relate to each other. The RRA is designed to contribute to an insider's perspective of the system; it does not reject or abandon the traditional methods and techniques of the social sciences but provides for ways to complement and enrich them (Beebe, 1995).

By early 1994, techniques considered as RRA were already evolving in various ways and spreading to at least 40 developing countries, including Botswana, Cameroon, Egypt, Ethiopia, India, Zambia and Zimbabwe (Chambers, 1994). RRA has also been spreading to Australia, Canada, Germany, Norway, Switzerland and the United Kingdom (Chambers, 1994). Much of the innovation has been in the non-governmental organization (NGO) sector, especially in India and Kenya, but there has been an increase in the adoption and adaption of the RRA methods by the government. There is a rising usage of the RRA methods by graduate students who are conducting their research in different higher education institutes, and university faculties have shown interest in over 20 countries (Chambers, 1994).

3.2 Materials and Methods

3.2.1 Study area

The study was carried out in the Mnisi community (Figure 3.1), which falls under the Mnisi Traditional Authority. The community consists of several villages, and the villages that were visited during the study include Clare A, Clare B, Welverdiend A, and Welverdiend B.

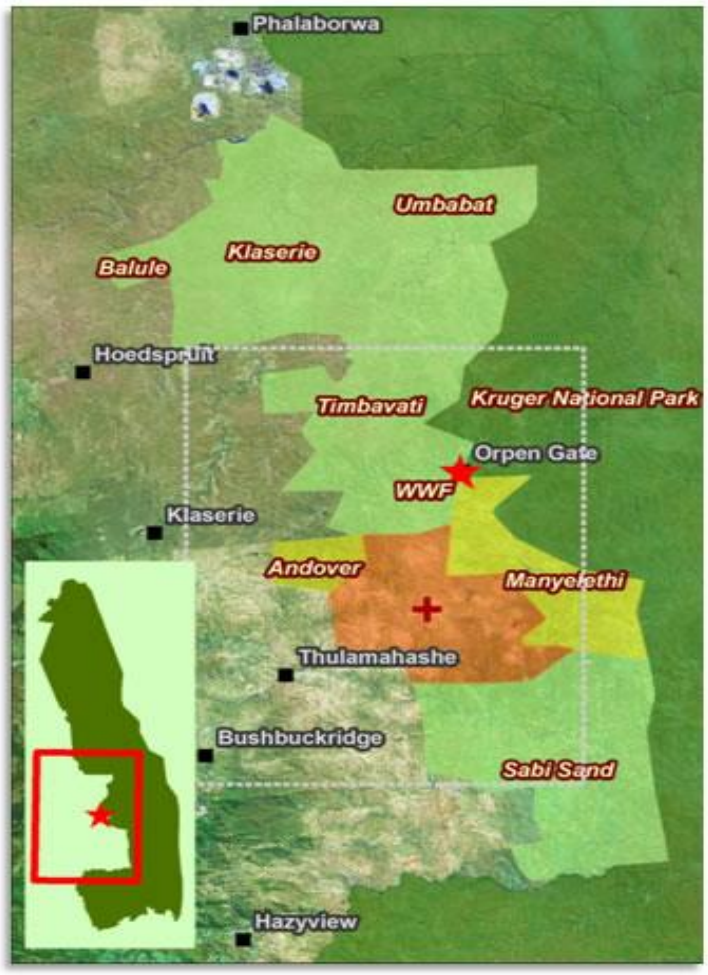

Figure 3.1: Map of the study area within the Bushbuckridge local municipal area (De Bruin, 2015)

3.2.2 Survey procedure

A meeting was held with the traditional chief and the dip tank committees of the local community to explain the purpose of the research and permission was granted for the survey. The survey was then carried out using the RRA approach where the information was obtained through interviews, general conversations, observations and field walks. The interviewed individuals consisted of local farmers (small-scale), animal health technicians, herdsmen and herbalists. Ethics permission was granted by the Faculty of Health Sciences; see Appendix 1. The interviews were conducted using a questionnaire (Appendix 2) that required information such as interviewee's personal information (sex, age, academic level and occupation), the type of EVM the interviewee uses, how it is acquired, stored, prepared, administered to the animals, how the animals are treated and monitored, and how the ethnoveterinary knowledge is shared amongst ethno-practitioners (Weckerle *et al*., 2018; Heinrich *et al*., 2018; Gakuubi and Wanzala, 2012).

The frequency index was calculated using the following formula:

FI = FC/N*100

where FC is the number of informants who mentioned the use of the species and N is the total number of individuals who participated in the study (50 in this study). The frequency index is high when there are many informants who mentioned a particular plant and low when there are few use reports (Madikizela *et al*., 2012).

Figure 3.2: Local dipping tanks at Mnisi community

The interviews were conducted at the local dipping tanks (Figure 3.2) with the help of an environmental monitor who also acted as an interpreter.

3.2.3 Plant collection

Figure 3.3: Plant collection in the local bush with the guidance of a respondent

Plant collection was done following Weckerle *et al*. (2018) where plants were collected under the guidance of respondents from various villages within the Mnisi community (Figure 3.3). Notes were taken during the conversations, and pictures of the plants were also taken along with the precise geographical coordinates of the plant. Plant material was collected for identification and for further laboratory investigations. Plant specimens were labelled, pressed and were identified at the HGWJ Schweickerdt Herbarium, University of Pretoria, where voucher specimens were deposited.

3.3 Results and Discussion

3.3.1 Informants

During the survey 50 people were interviewed (Figure 3.5) at five different dipping tanks in the Mnisi community. The interviewees consisted mostly of farmers and some herdsman. Out of the 50 interviewees, 10 were female farmers (Table 3.1). Most (31) individuals had knowledge about EVM while the remaining 18 individuals had no knowledge, and thus the information was acquired from 31 informants (Figure 3.6). The majority (32) of individuals

were above 50 years while the remaining 18 ranged in age from 18 to 49. All the participants had a mean age average of 54.84 and the individuals with knowledge about EVM ranged from 40-83 years. Most respondents were unemployed and depended on the cattle trade for income (buying and selling) while some were self-employed. The majority (90%) of the interviewees grew up in the study area.

Figure 3.4: Interviews with the local people at the dipping tanks in the Mnisi community

Table 3.1: Age groups of all interviewees in the Mnisi community

	Age groups of all interviewees				
	15-30	31-45	46-60	61-75	76-90
MALE	4	8	8	13	6
FEMALE	1	4	1	4	1

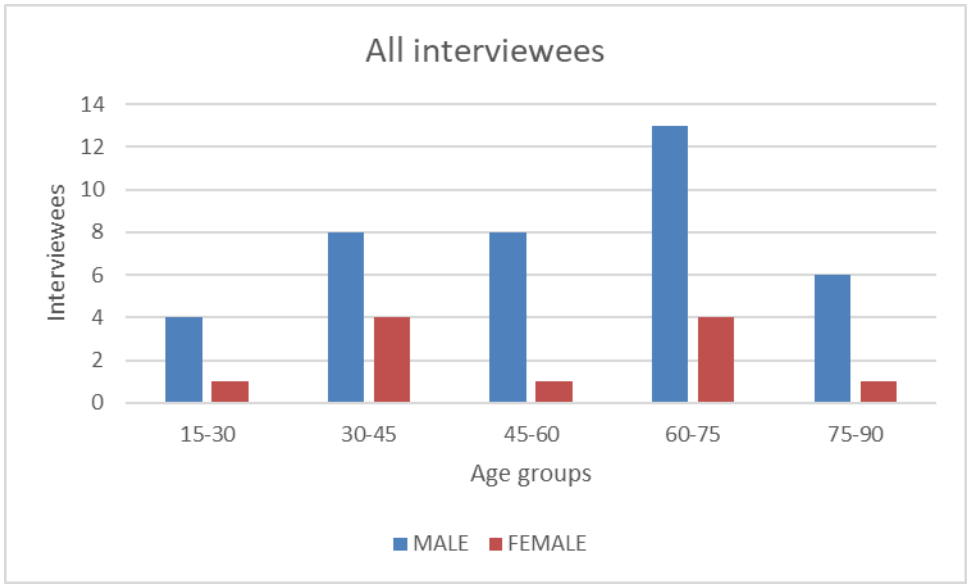

Figure 3.5: Age groups of all interviewed individuals

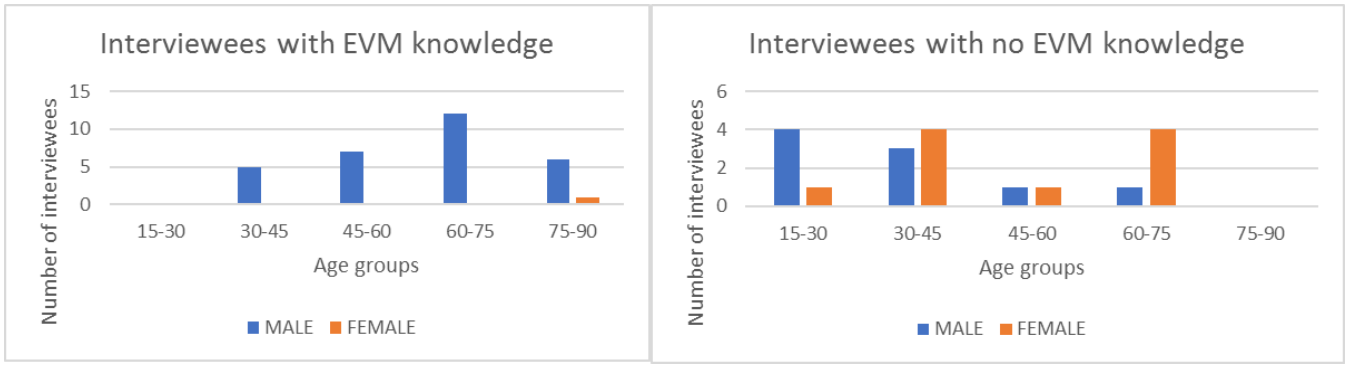

Figure 3.6: Interviewees with EVM knowledge vs interviewees with no EVM knowledge

Similar results were obtained in the surveys done by Luseba and Van der Merwe (2006) and Maphosa and Masika (2010), where males from the age of 40 were the most knowledgeable age group when recording the use of plants as ethnoveterinary medicine. The younger generation had no knowledge about EVM which is most likely due to lack of interest and migration to urban areas (Luseba and Van der Merwe, 2006). Respondents mentioned that, in their culture, males were often responsible for the well-being of the livestock and hence females lacked knowledge about using EVM. The local animal technician did not have knowledge about the EVM practices and traditional healers did not share their EVM knowledge with the farmers, hence the farmers did not consult traditional healers for animal healthcare. Similar reports were given for Venda farmers and Tsonga farmers of Greater Giyani (Luseba and Van der Merwe, 2006; Luseba and Tshisikhawe, 2013)

3.3.2 Diseases affecting the community livestock

The Mnisi community livestock is continually at risk of contracting different diseases due to its proximity to wildlife, and the community was recently suffering from a Foot and Mouth Disease (FMD) outbreak in cattle. Due to their value, cattle are kept as a status symbol amongst these ethnic groups. In a similar fashion to the Tswana and Venda populations, cattle were the animals most predominantly treated in the Mnisi community, hence the diseases mentioned were primarily for cattle (Luseba and Tshisikhawe, 2013; Van der Merwe *et al.*, 2001). Diseases mentioned during the interviews included diarrhoea, black quarter, gall disease, foot and mouth disease, eye infections, swollen stomach, sudden weight loss and wounds. Of the informants, 35% mentioned diarrhoea as a prevalent disease within the community. Diarrhoea was believed to be caused by intestinal parasites and was suspected when the cattle lacked appetite, were weak or secreted watery faeces. Similar results and observations were reported by Luseba and Tshisikhawe (2013). According to the farmers, gall disease symptoms could not be clearly identified and were often associated with those of diarrhoea. The farmers also mentioned that a swollen gallbladder was evidence of the disease. In veterinary terms, gall sickness refers to the tick-borne disease anaplasmosis (caused by *Anaplasma marginale*). However, an enlarged gallbladder can result from non-specified illness that result in inability of the animals to eat, which may be caused by a number of ailments. Many (58%) of the farmers mentioned that black quarter was also prevalent in the area and this disease was suspected when a cow had a swollen limping hind-leg or was semi-paralyzed. Wounds were caused by external cuts from animal fights, thorns and ticks etc. Cattle were the most affected animals hence they were the most commonly treated. Farmers reported that the same plant remedies were seldom used for sheep and goats as they did not usually get affected by the same diseases as cattle, or to the same extent.

3.3.3 Plants used as ethnoveterinary medicine

During the survey, eleven plant species belonging to seven families were reported (Table 3.2). The family with the most plant species was the Fabaceae with five plant species. This family was also one of the top three most frequently mentioned plant species, for example *Elephantorrhiza obliqua* Burtt Davy which was mentioned by 48% of the respondents for the same disease. *Aloe marlothii* A. Berger from the Asphodelaceae family followed with 39% of people mentioning it as being used, along with *Senna italica* Mill. from the Fabaceae with 19% of people reporting its use. The Fabaceae family is one of the most used plant families in EVM amongst different ethnic groups in South Africa. It is the second largest family of plants in South Africa, consisting of over 490 plants (Luseba and Tshisikhawe, 2013).

Luseba and Tshisikhawe (2013) reported that the Fabaceae was the predominant family of plants used amongst Venda-speaking people and similar results were reported by Van der Merwe *et al*. (2001) for Tswana-speaking people

Table 3.2: Plants used in ethnoveterinary medicine followed by the treated diseases, plant parts, administration, voucher specimen numbers and the frequency index.

Family and Scientific Name	**Vernacular name**	**Disease**	**Plant part**	**Preparation**	**Administration**	**Voucher specimen number**	**Frequency Index**
Asphodelaceae *Aloe marlothii* A.Berger	Mahgana	Gall and diarrhoea	Leaves	Infusion (cut half of the aloe leaf then slice it to smaller pieces and soak in water overnight)	Orally using a 1l bottle for adults and 500 ml for calves	PRU 124370	22%
Fabaceae *Albizia* sp.	Xisitana	Swollen stomach	Roots	Root skin infused in water and left overnight (infusion)	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124379	2%
Vitaceae *Cissus quandrangularis* L.	Nyangala	Wounds	Stem	Grind the stem and apply the juice directly on the wound	Applied directly on the wound (enough to cover the wound)	PRU 124377	14%

Peraceae *Clutia pulchella* L.	Mjamonti	Gall	Bark	Bark boiled in water	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124369	2%
Fabaceae *Elephantorrhiza obliqua* Burtt Davy	Xixengani	Diarrhoea	Roots	Slice the roots and boil in water (Decoction) some slice the roots and infuse in water overnight	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124373	24%
Celastraceae *Gymnosporia* sp.	Xihlangwa	Black quarter and diarrhoea	Roots	Root skin infused in water and left overnight (infusion)	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124372	2%
Pedaliaceae *Harpagophytum procumbens* (Burch.) DC. ex Meisn.	Ntjolovoti	Diarrhoea, black quarter, if the cow is not eating or ruminating	Roots	Chopped roots are infused in water over night	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124378	2%

Fabaceae *Philenoptera violacea* (Klotzsch) Schrire	Mbhandzu	Gall, diarrhoea and general ailments	Bark	Ground bark infused in water over night	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124375	2%
Fabaceae *Schotia brachypetala* Sond.	Chochela-mandleni	Foot and mouth disease, black quarter and general ailments	Bark	Ground bark boiled in water	Orally using a 1L bottle for adults and 500ml for calves	PRU 234371	2%
Fabaceae *Senna italica* Mill.	Xintomane	General ailments	Roots	Roots boiled in water	Orally using a 1L bottle for adults and 500 ml for calves	PRU 124374	2%
Euphorbiaceae *Synadenium grantii* Hook.f.	Mdleve	Eye problems	Stem	Milky sap applied directly on the area between the eye and the ear (just above the eye)	Apply directly	PRU 124376	8%

According to the respondents in this survey, knowledge of ethnoveterinary practices is passed on from generation to generation orally and the information is culture specific. Locality also influences the plants used to prepare the remedies because some plants might not be available in certain regions. Similar results were reported by Luseba and Tshisikhawe (2013), Van der Merwe *et al*. (2001) and Luseba and Van der Merwe (2006). Farmers preferred to use plants that were used or recommended by family members or other farmers of the same ethnic group in the same locality. Luseba and Tshisikhawe (2013) reported similar results. Luseba and Van der Merwe (2006) conducted a similar study with Tsonga people in the Greater Giyani municipality and it was found that the famers used 38 plant species from 19 families. This was different from the farmers in the Mnisi community who used fewer plants, perhaps because the area is smaller compared to Giyani although both studies were done amongst Tsonga people. The compared results showed that it was also possible for the same ethnic groups in different localities to use the same plants, for example people in Giyani and those in the Mnisi community both used *Senna italica* (Xintomani) for diarrhoea and *Cissus quandrangularis* (Nyangala) for wounds.

Mnisi farmers used *Elephantorrhiza obliqua* (Xixengani) for diarrhoea and black quarter while Giyani farmers used *Elephantorrhiza elephantina* (Xixuvari) for black quarter and as an appetite stimulant. *Synadenium grantii* (Mdleve) was used by the Mnisi farmers for eye infections while *Synadenium cupulare* was used by the Giyani farmers for the treatment of eye infections.

3.3.4 Plant remedy preparation and administration

Remedies were prepared from roots, bark, stems and leaves, as also recorded by Luseba and Van der Merwe (2006). The most frequently used plant part was the roots followed by the bark, stem and then leaves. Most (84%) of the farmers in the Mnisi community used single plants to prepare the remedies. Similarly, 68% of the Tswana people also used single plants (Van der Merwe *et al*., 2001). Water was used as an extraction solvent to prepare the plant remedies and the common methods of preparation were decoctions and infusions. These methods were also used by the Venda (Luseba and Tshisikhawe, 2013), Tswana (Van der Merwe *et al*., 2001), Xhosa (Maphosa and Masika, 2010; Masika *et al*., 2000) and Zulu (McGaw and Eloff, 2008). Decoctions were prepared by boiling the plant material in water for a certain period, and infusions were prepared by soaking the plant material in water, usually overnight. Other methods of preparation included the mashing of fresh plant parts to form pastes while other remedies were prepared by pressing the sap out of the fresh plant part and using the sap for treatment, which was also reported by Masika *et al*. (2000).

Similarly to the Venda and Tswana people, the Mnisi community people (Tsonga) believe that wild plants are more potent than cultivated plants thus they prefer to use fresh plant material collected from the wild. Therefore plants were not collected for storage or planted at private homes but only collected when they were needed which was supported by earlier reports (Luseba and Tshisikhawe, 2013).

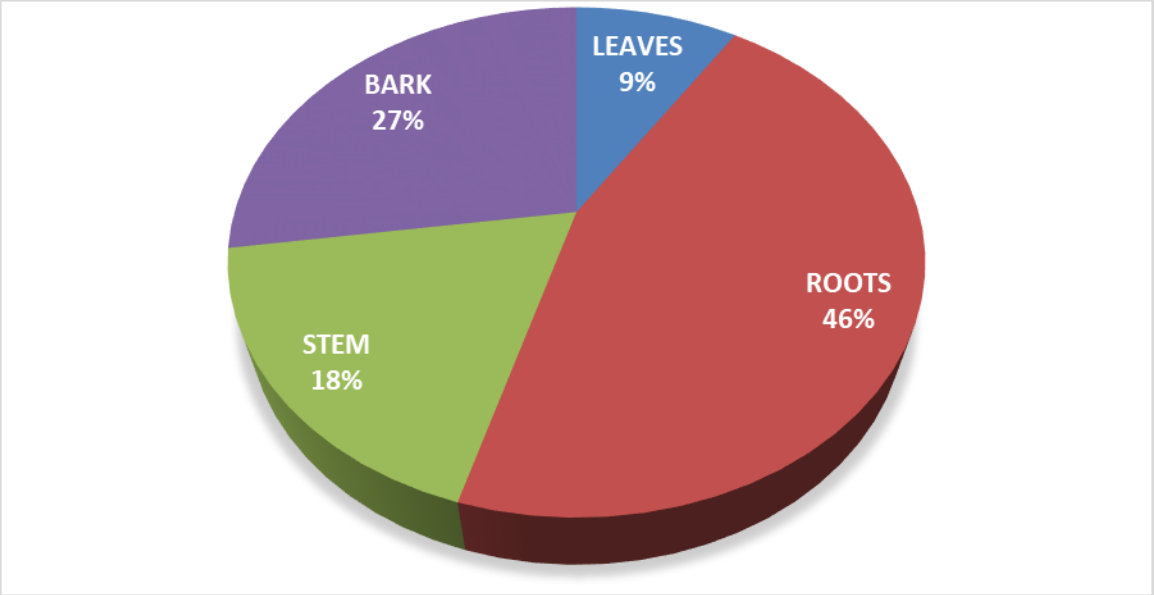

Figure 3.6: Plant parts used to prepare EVM by the farmers of the Mnisi community

The plants were regularly available in the study area. One of the reasons for this is that the community only harvested half of each plant part during plant collection to ensure future availability. Water-based remedies were administered orally using a beverage bottle with a capacity of 1L for adult cows and 500 ml for calves. Skin conditions such as wounds, sores, cuts and warts were treated by directly applying a mashed plant like *Cissus quadrangularis* (aerial parts) directly on the infected area as a poultice. Luseba and Van der Merwe (2006) also reported that the Tsonga people of Greater Giyani also used the same plant for wound treatment, as a tick repellent and for lumpy skin disease. For eye infections, plants like *Synadenium grantii* are cut open and squeezed to release milky sap which is then applied topically on the area which was referred to as the “nerve” just outside the eye. The sap is carefully applied so that it does not enter the eye as it may cause damage to the eye. Luseba and Van der Merwe (2006) reported that the same plant was also used to treat black quarter by applying the latex/sap on the limping leg.

3.3.5 Other remedies used as ethnoveterinary medicine

Some farmers used non-plant material to treat animal infections, for example to treat black quarter the famers frequently used a hot iron/steel spear to pinch the cows on the infected limbs. This was said to allow excess blood to escape as black quarter was suspected when there was limping or swelling of legs. Salt was also mixed with other plants to treat different diseases including gastrointestinal diseases. For example some farmers added salt to *Aloe marlothii* infusions to treat diarrhoea and gall sickness. The use of salt by the Venda and Tswana people was also reported (Luseba and Tshisikhawe, 2013). Other farmers mentioned the use of Jeyes fluid as a tick repellent and petrol for wound disinfection.

3.4 Conclusion

This study provides evidence that the use of plant remedies to treat prevalent livestock diseases is still common amongst the people of the Mnisi community in the Bushbuckridge municipality. This is due to the low cost, easy access and easy administration of the remedies. However, it is evident that similarly to the Tsonga people at Greater Giyani, the Tsonga people in the Mnisi community use EVM to a lesser extent compared to the Tswana, Venda and Xhosa people (Luseba and Van der Merwe, 2006). Although there are similarities in plants that are used by different ethnic groups, EVM still remains specific to ethnicity and locality. Women and young people possessed less knowledge about EVM. The relaying of EVM knowledge from one generation to the next encounters difficulties as young people move to urban areas to seek job opportunities, thus it is important to document EVM knowledge while it is still freely available. Feedback sessions with women and children are important to disseminate the knowledge and to make recommendations. It is important to do more studies in other regions to compare the practices amongst ethnic groups of different regions. There is a growing concern regarding the toxicity of the remedies and thus more research also needs to be done on the pharmacological activities of the plants to provide more information about the dosing and concentration of the remedies.

Chapter 4

Antimicrobial activity of crude extracts against bacterial, fungal and mycobacterial pathogens.

4.1 Introduction

Antimicrobial resistance is referred to as the ability of microorganisms to withstand an active dosage of an antimicrobial agent. The resistance occurs when the microbes transform in a way that decreases the effectiveness of drugs, chemical compounds, or other agents designed to cure or prevent microbial infections (Khan *et al.*, 2015). Antimicrobial resistance is mainly caused by the misuse/overuse of antimicrobials (antibiotics, antifungals, antivirals etc). This normally happens when patients take antimicrobials for shorter periods (don't complete the course), at a lower dose or for diseases caused by a different microorganism (Ben-Ami *et al*., 2012; Kaikabo *et al*., 2009). The inappropriate use of antimicrobial agents promotes the development of antibiotic-resistant bacteria, antifungal-resistant fungi and antimycobial-resistant mycobacteria (Madikizela and McGaw, 2018; Mérillon, 2018). Every time a patient takes antibiotics, susceptible bacteria (bacteria that antibiotics can still attack) are killed, but resistant bacteria survive and continue to grow and multiply. This is how the repeated use of antibiotics increases the number of drug-resistant bacteria same applies for fungi and mycobacteria (Khan *et al.*, 2015; Valabhaneni *et al*., 2015; Thoen *et al*., 2006). Examples of antibiotic-resistant strains include vancomycin-resistant and methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, carbapenem-resistant *Pseudomonas aeruginosa* and cephalothin-resistant *E. coli* (Malema, *et al*., 2018; Khachatourians, 1998). Some *Candida* species such as *C. albicans* are already demonstrating increasing resistance to various antifungal agents such as fluconazole and the echinocandins (anidulafungin, caspofungin, and micafungin), which are considered to be the first and second line of antifungal drugs (Howard *et al*., 2010). Fluconazole and echinocandin resistant *Candida* infections have few and limited treatment options remaining (Ben-Ami *et al*., 2012; Howard *et al*., 2010; Mortensen *et al*., 2010; Verweij *et al*., 2009).

Resistance may also be as a result of agricultural use of antimicrobials for example using fungicides to treat crop diseases. The use of azole fungicides which are similar to azole medications like fluconazole can lead to resistant strains of *Aspergillus* species in soil and other environmental niches (Ben-Ami *et al*., 2012). Depending on location it is estimated that up to 12% of *Aspergillus* infections are resistant to antifungal medications (Ben-Ami *et al*., 2012). The use of antimicrobials in animals has played a major role in the emergence of

resistant strains resulting in animals being major reservoirs of antimicrobial resistant strains (Aarestrup *et al*., 2008). The levels and patterns of resistance observed in food animals are due to extensive usage of antimicrobial drugs, resulting in the spread of resistant genes between animals. In particular, vertical spread between the generations is significant, such as the spread of resistant *Salmonella* in the poultry and swine breeding industries and successful adaptation of resistant strains to the animal reservoir (e.g., MRSA CC398). The extensive usage of antibiotics includes feeding animals with growth promoters, which exposes the bacteria to sub-lethal concentrations of drugs over long periods, resulting in the bacteria gaining resistance (Malema *et al*., 2018; Khan *et al.*, 2015).

Microbial species are able to transfer resistant DNA sequences amongst each other. The transfer is not only between bacterial species but can also happen between different ecological niches such as humans and ruminants (Kaikabo *et al*., 2009; Khachatourians, 1998). For example the addition of tylosin in animal feed has resulted in the development of erythromycin-resistant streptococci and staphylococci, not only in the animals but also in their caretakers (Khachatourians, 1998). *Mycobacterium bovis* causes bovine TB which is a zoonotic disease that is able to spread to humans if there is contact with infected cattle or contaminated products such as the ingestion of unprocessed milk or inhalation of infectious droplet nuclei, thus resistant *M. bovis* can be easily passed on from animals to humans (McGaw, *et al*., 2008; Thoen *et al*., 2006).

There are significant structural similarities between antibiotics which are used to treat humans and those used in veterinary therapy, which results in the transfer of resistance between animals and humans (Van Vuuren and Holl, 2017). For example resistance to ormetoprim, an animal antibiotic, may result in resistance to trimethoprim used for human therapy as they have similar chemical structures (Cos *et al*., 2006; Khachatourians, 1998). Also in Great Britain it was found that the treatment of pigs and calves with both apramycin (used for animals) and gentamicin (used for both animals and humans) resulted in antibiotic-resistant *E. coli* found in the pig farmer. It was also found that the gentamicin-resistant strains isolated from human clinical samples were resistant to apramycin (Khachatourians, 1998).

Drug development is slow while microbial resistance is increasing fast, thus there is an urgent need to look for new antimicrobial agents from alternative sources such as plants as they contain various chemical compounds which may be highly active against opportunistic microbes and may also have lower toxicity towards mammalian cells. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug development because of their unmatched chemical diversity (Cos *et al*., 2006).

Hence it is important to investigate the plants growing in the different ecosystems of South Africa as they produce a variety of secondary metabolites. The aim of the study was to evaluate and compare the antimicrobial efficacy of aqueous and organic solvent extracts of selected plants crude extracts.

4.2 Materials and Methods

4.2.1 Plant selection and extraction.

Traditional methods were used for plant extraction as described or prepared by the respondents. Organic solvents were used in order to compare the traditional and organic solvent methods of extract preparation. Three plant species that were frequently cited and not well-investigated for bioactivity from a preliminary literature search were selected and analysed for their biological activity. Plant material that was used included roots of *Elephantorrhiza obliqua*, bark of *Schotia brachypetala* and leaves of *Aloe marlothii*. Fresh plant material was boiled with water to prepare decoctions while other fresh plant material was suspended into water to form infusions. Water extracts were also prepared with 1 g of powdered plant material extracted with 10 ml of distilled water (aqueous). Acetone extracts were prepared in the same way in a ratio of 1:10 (plant material: acetone) using technical grade acetone (Merck). Acetone is the best choice of an organic extractant due to its ability to solubilize antimicrobial substances from plants, its low toxicity to bioassays and it is also easy to remove from extracts thus acetone was selected for extraction (Eloff, 1998a). The extracts were shaken on a Labotec model 20.2 shaker for 30-60 min and centrifuged at 1372 xg for 5 min. Extraction was repeated three times on the plant material. The three supernatants were combined and dried in pre-weighed vials. Samples were prepared in concentrations of 10 mg/ml and were resuspended in the same solvents used for extraction (acetone and water).

4.2.2 Microbial strains

According to McGaw and Eloff (2010) the National Committee for Clinical Laboratory Standards (NCCLS, now CLSI or Clinical and Laboratory Standards Institute) recommended that Gram-positive *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) be used for routine antibacterial screening, thus in this study all these microorganisms were used to test antimicrobial activity. The strains were maintained in Muller Hinton (MH) agar at 37°C, cultured in Muller Hinton broth and incubated for 12 hours at 37°C before screening.

In this study three fungal species were used to test the antifungal activity of the plant extracts, namely the yeast species *Candida albicans* and *Cryptococcus neoformans* as well as *Aspergillus fumigatus* (a mould). Clinical isolates of the species were obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. All the fungal isolates were maintained at 4°C on Sabouraud Dextrose (SD) Agar.

*M*ycobacterium *tuberculosis* ATCC 25177 and *M*. *bovis* ATCC 27290 were the two pathogenic *Mycobacterium* strains from the American Type Culture Collection (ATCC) that were used to evaluate the antimycobacterial activity of selected extracts. *M. tuberculosis* ATCC 25177 was maintained on Lowenstein-Jensen (LJ) slants supplemented with glycerol, while *M. bovis* ATCC 27290 was maintained on LJ slants supplemented with pyruvate.

4.2.3 Antibacterial assay to determine the minimum inhibitory concentration (MIC)

The serial microplate dilution method developed by Eloff (1998b) was used to determine the minimal inhibitory concentration (MIC) values of the plant extracts against each bacterium. Two-fold serial dilutions were prepared in 96-well microtitre plates as follows: sterile distilled water (100 µl) was placed in each well using a micropipette and 100 µl of a 10 mg/ml plant extract was added to each of the first wells of the relevant series of dilutions, resulting in the extract being diluted to 50%. One hundred microliters was then removed and placed into the next well. The process was repeated all the way to the bottom of the plate with 100 µl from the last rows being discarded to ensure that all wells contain the same amount of extract. Exactly 100 µl of overnight cultured bacteria was added in each well. Then, 40 µl of *p*-iodonitrotetrazolium (INT) (0.2 mg/ml) dissolved in water was added to each well. The plates were incubated for 18 h at 37°C in a humidified atmosphere. The minimal inhibitory concentration was recorded as the lowest concentration of the extract that inhibits growth. The reduction of INT to a red formazan indicated bacterial growth so inhibition of growth resulted in lower intensity of the red colour. Gentamicin was used as positive control and the solvent (acetone or water) as negative control (Eloff, 1998b). The extracts were tested in triplicate and the entire experiment was repeated.

4.2.4 Biofilm inhibition activity of the crude extracts against bacterial strain *Enterococcus faecalis*

The biofilm inhibition assay was done according to Sandasi *et al*. (2011). Different stages of biofilm development were investigated in this study which included: no attachment (T0, where plant extract was added immediately after adding bacteria to the wells of the microplate) and irreversible attachment (T24, where plant extract was added after 24 hours of bacterial incubation). A 100 µl aliquot of standardized concentration of *E. faecalis* ATCC 29212 cultures ($1x10^6$ CFU/ml) was added into wells of microtitre plates and incubated at

37°C for 0 and 24 hours without shaking. After the selected incubation periods, extracts (100 µl of 1 mg/ml concentrations) were added. Gentamicin (1 mg/ml) was used as a positive control while acetone and sterile distilled water were used as negative controls.

The biofilm biomass was assessed using the crystal violet staining assay (Sandasi *et al*. 2011). Following incubation the plates were washed three times with distilled water, then oven-dried at 60°C for 45 min. After drying, the wells were stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were then washed three times with sterile distilled water to remove unabsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 125µl of ethanol to distain the wells. One hundred microliters (100 µl) of the distaining solution was then transferred to a new plate and the absorbance determined at 590 nm using a micro-plate reader (Universal micro-plate reader ELX 800). The mean absorbance of the samples were determined and percentage inhibition was calculated following the above equation (Sandasi *et al.*, 2011).

$$\text{Percentage inhibition} = \frac{{}^{OD}\text{Negative control} - {}^{D}\text{Experimental}}{{}^{OD}\text{Negative control}} * 100$$

4.2.5 Antifungal assay to determine the minimum inhibitory concentration (MIC)

The serial microplate dilution method developed by Eloff (1998b) modified by Masoko and Eloff (2005) was used to determine the minimal inhibitory concentration (MIC) values for plant extracts against fungal strains. All wells of sterile 96-well micro plates were filled with 100 µl of sterile distilled water. Exactly 100 µl of a 10 mg/ml plant extract was added in each of the first wells and serially diluted two-fold down the column to the last well. Exactly 100 µl of fungal cultures ($1x10^5$ CFU/ml) grown in SD broth were added to each well. Amphotericin B was used as a positive control. As an indicator of growth 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium (INT) was dissolved in sterile distilled water and added to each well. The plates were incubated for 24 h at 37°C and 100% relative humidity. The MIC was recorded as the lowest concentration of extracts that could inhibit fungal growth after 24-48 h. The extracts were tested in triplicate and the entire experiment was repeated.

4.2.6 Antimycobacterial assay to determine the minimum inhibitory concentration (MIC)

Permission to conduct the study was granted by the Department of Agriculture, Forestry and Fisheries of South Africa under Section 20 of the Animal Diseases Act. The pathogenic *Mycobacterium* strains were handled following the WHO (2012) biosafety standard which includes working in a biosafety level 2+ laboratory, wearing protective gear, following the required process for the decontamination and disposal of waste. All the experiments were performed in the laboratory approved by the Institutional Biosafety Committee for Mycobacterial Cultures by University of Pretoria, in the Department of Veterinary Tropical Diseases. *M*. *tuberculosis* ATCC 25177 and *M*. *bovis* ATCC 27290 were the two pathogenic *Mycobacterium* strains from the American Type Culture Collection (ATCC) that were used to evaluate the antimycobacterial activity of selected extracts. Dr Balungile Madikizela assisted with conducting the antimycobacterial assays. *M. tuberculosis* ATCC 25177 was maintained on Lowenstein-Jensen (LJ) slants supplemented with glycerol, whereas *M. bovis* ATCC 27290 was maintained on LJ slants supplemented with pyruvate. Both strains were maintained for a month and colonies of each culture were transferred into 3 ml of supplemented Middlebrook broth, homogenized by vortexing, allowing larger particles to settle. Supplemented Middlebrook broth was used to prepare the test inoculum, which was then adjusted to match the McFarland standard 1 equivalent to 3.0×10^8 diluted to a final density of 5×10^5 CFU/ml in the medium. The MIC values of the six plant extracts were determined using the micro-dilution assay according to Eloff (1998b) and Jadaun *et al*. (2007) in a 96 well microtitre plate. Samples were prepared at a concentration of 10 mg/ml in 10% dimethylsulfoxide (DMSO) and water for water extracts, and then serially diluted with OADC-supplemented Middlebrook 7H9 broth twofold (100 µl) down to the last wells of the microtitre plate. Isoniazid, streptomycin and rifampicin were used as positive controls, whereas 10% DMSO, water, inoculum and OADC-supplemented Middlebrook 7H9 broth were negative controls. Exactly 100 µl of mycobacterial cultures were added in all the wells. To avoid contamination plates were sealed with parafilm placed into plastic bags and incubated for 7-10 days at 37°C. After incubation, 40 µl of 0.2 mg/ml of freshly prepared p-iodonitro-tetrazolium chloride (INT) solution was added to each each well in order to determine the MIC. Colour detection after the addition of INT was read as soon as colour became visible in the untreated control wells. The reduced colour formation indicated the inhibition of mycobacterial growth. Concentrations were tested in triplicate and the experiments were repeated twice.

4.3 Results

4.3.1 Minimum inhibitory concentration against bacteria

Nine extracts from three plants were tested against four bacterial species and three fungal species. Six extracts were selected from the nine extracts and tested against and two mycobacterial species and the MIC values are presented in **Table 4.1**.The plants were used traditionally by the Mnisi community to treat diarrhoea, gall, foot and mouth disease, black quarter and general ailments. Some of the plant extracts were traditionally prepared as infusions and decoctions as described by the respondents while some were prepared with an organic solvent (acetone).The extracts were prepared from plant parts including leaves, bark and roots depending on the plant part used. Plant crude extracts were considered to be promising for further investigation if they have MIC values against a microbial test organism below 100 µg/ml (0.1 mg/ml) (Eloff 2004; Rios and Recio, 2005).

Table 4.1: Antimicrobial activity of nine extracts from three plant species against bacterial, fungal and mycobacterial pathogens.

Samples	MIC values (mg/ml) and microorganisms								
	Ec	Ef	Pa	Sa	Ca	Cn	Af	Mt	Mb
S. brachypetala									
Acetone	0.63	0.11	0.32	1.25	2.50	1.63	0.63	0.035	0.04
Decoction	2.08	0.24	2.50	2.5	2.50	1.44	1.25		
Aqueous	1.04	0.63	1.67	1.25	1.63	0.63	0.82	0.02	0.04
E. obliqua									
Acetone	0.27	0.14	0.09	0.63	2.50	2.07	0.63	0.32	0.32
Decoction	1.04	0.22	0.34	1.25	2.50	1.63	0.03	0.32	0.16
Infusion	1.04	0.22	0.44	0.84	2.50	2.07	0.04	0.16	0.08
Aqueous	0.63	0.32	0.37	0.63	2.50	2.07	0.02	-	-
A. marlothii									
Acetone	0.21	0.13	0.13	0.19	1.25	0.81	1.20	0.63	1.2
Infusion	0.68	0.43	0.87	0.73	2.50	1.63	2.07	-	-
Gentamicin	0,08	0,02	0,02	0,04	-	-	-	-	-
Amphotericin B	-	-	-	-	0.16	0.02	0.04	-	-
Streptomycin	-	-	-	-	-	-	-	0,16	0,02
Rifampicin	-	-	-	-	-	-	-	0,02	0,01

Isoniazid	-	-	-	-	-	-	-	0,63	0,04

Minimum inhibitory concentration (MIC), *Escherichia coli (Ec), Enterococcus faecalis (*Ef*)*, *Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* (Sa), *Candida albicans (*Ca*), Cryptococcus neoformans (*Cn*)* and *Aspergillus fumigatus (*Af*)*, *Mycobacterium tuberculosis (*Mt*)* and *Mycobacterium bovis (*Mb). Standard deviation in all cases was 0.

4.3.2 Biofilm activity

The biofilm inhibitory activity (BIA) of the crude extracts against pathogenic *Enterococcus faecalis* is represented by Figure 4 below. Out of all the tested bacterial strains the plant extracts had more antibacterial activity against planktonic *E. faecalis* which is known to inhabit the gastrointestinal tracts of both humans and mammals. Thus it is also important to evaluate the activity of the plants against *E. faecalis* biofilm.The four extracts were chosen in this study due to their antibacterial activity (MIC) against planktonic *E. faecalis*. Only two biofilm development stages were investigated which were T0 and T24. According to Sandasi *et al*. (2008) extracts above 50% are considered to have good biofilm inhibitory properties. While extracts below 50% were considered to have poor BIA, extracts with % below zero were considered to enhance the biofilm growth.

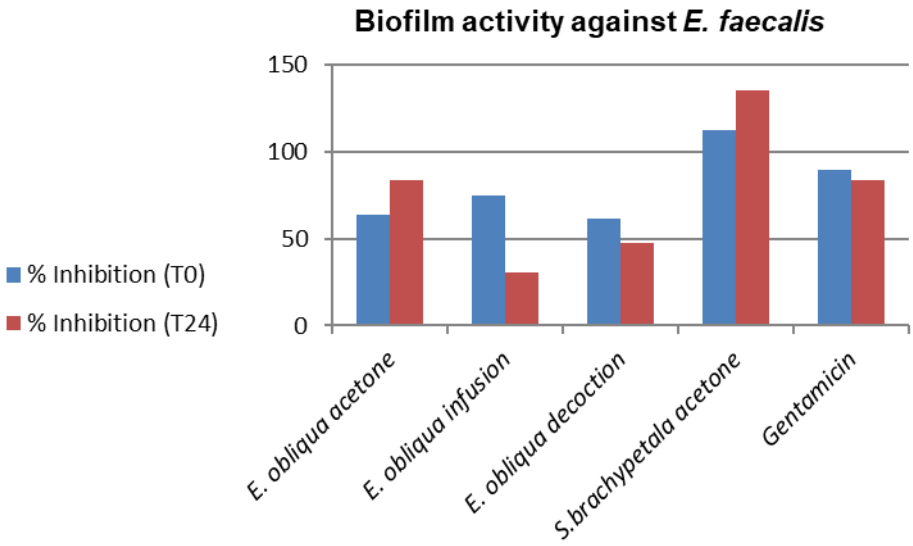

Figure 4: Inhibitory percentage of four plant extracts against *E. faecalis* biofilm at 0 hour and 24 hours.

4.4 Discussion

All the tested plant extracts had MIC values ranging from 0.02 to 2.50 mg/ml against all microbial strains. Out of all the tested samples the acetone extracts had better antibacterial

activity with MIC values ranging from as low as 0.09 to 0.27 mg/ml compared to the samples extracted using traditional methods (decoctions and infusions). However water extracts had the highest antifungal activity with MIC values ranging from 0.02-2.50 mg/ml compared to organic solvent extracts which had MIC values ranging from 0.63-2.50 mg/ml. Water extracts also had the highest antimycobacterial activity compared to acetone with MIC values ranging from 0.02 to 1.2 mg/ml. The results indicate that relatively polar compounds that are extracted by water may be responsible for the antifungal and antimycobacterial activity. Similarly in a study done by Ramadwa *et al*. (2017) acetone crude extracts were found to have lower antifungal activity than the aqueous extracts against the three opportunistic fungal pathogens (*A. fumigatus, C. albicans, C. neoformans*).

E. obliqua extracts seemed to be the most active as *E. obliqua* acetone extract had the best antibacterial activity with an MIC value of 0.09 mg/ml against *P. aeruginosa*, while *E. obliqua* water extracts had the highest antifungal activity with MIC values of 0.02- 0.04 mg/ml against *A. fumigatus* respectively. However for antimycobacterial activity, *S. brachypetala* aqueous extract had the highest activity (MIC = 0.02 mg/ml). Since there are no reports yet on the biological activity of *E. obliqua* it is important to investigate its activity further as well as that of other species from the same genus. In a study by Mabona *et al*. (2013) water extracts prepared from leaves of *Elephantorrhiza elephantina* had little or no activity with MIC value of 16 mg/ml. In another study by Mathabe *et al*. (2006) it was found that water extracts prepared from *E. elephantina* (stem rhizome) and *Elephantorrhiza burkei* (stem rhizome) had MIC values of 0.10 mg/ml against *S. aureus* which is better activity compared to that of the root extracts in the present study (Table 4.1).

It is very interesting that *E. obliqua* aqueous and decoction extracts had better activity than the positive control amphotericin B against *A. fumigatus.* The low activity of amphotericin B against *A. fumigatus* and *C. albicans* may occur as a result of ergosterol biosynthesis reduction or formation of alternative sterols with reduced affinity for amphotericin B in the fungal cell membrane (Odds *et al.*, 2003). *S. brachypetala* acetone and water extracts also had better activity than the positive controls streptomycin and isoniazid, especially against *M. tuberculosis.*

E. faecalis is one of the biggest causes of nosocomial infections globally. Diseases associated with *E. faecalis* are serious and life threatening. The diseases include urinary tract infections, endocarditis, intra-abdominal and pelvic infections, catheter-related infections, surgical wound infections, and central nervous system infections (Mohamed and Huang, 2007). Biofilm communities are known to express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents (Sandasi *et al*., 2011).

In Table 4.1 of the present study acetone extracts had better antibacterial activity against planktonic *E. faecalis* compared to water extracts. The same trend can be observed in Figure 4 where acetone extracts had the highest biofilm inhibitory activity at both T0 and T24. *S. brachypetala* acetone extracts had highest inhibition of 113% at T0 and 135% at T24, while *E. obliqua* acetone extracts inhibit biofilm by of 64% at T0 and 83% at T24. Although *E. obliqua* water extracts showed activity at T0 with 74% inhibition, the activity decreased to 30% at T24. However the percentage inhibition of acetone extracts increased from T0 to T24 meaning that as the biofilm develops with time the inhibition properties of the extracts also increases. These properties might inhibit the *E. faecalis* cells from attaching to the biofilm and also prevent the biofilm from developing into a matured stage.

4.5 Conclusion

Although traditionally prepared extracts did not yield highly active antibacterial properties, the extracts showed significant antifungal and antimycobacterial activities. This is very promising considering that local people rely on water as a solvent to prepare the remedies. It is interesting that some of the extracts had better activity than the positive controls such as amphotericin B which is currently used as one of the main defence pharmaceuticals against antifungal pathogens. This suggests that the extracts should be further investigated as they might contain compounds that are essential for the development of new antimicrobial products. Therefore, plant species with potential antibiotic activity such as *E. obliqua,* which has not been previously investigated, should be subjected to further studies including isolation of antimicrobial compounds. Although some extracts showed moderate activity it must be noted that there are different compounds within an extract that may act in synergy, but once isolated, they might demonstrate more activity than the crude extract. Therefore, moderate antimicrobial activity of crude extracts is worthy of further investigation. It is also important to study the toxicity of the plants thus Chapter 5 of this study investigates the *in vitro* toxicity along with the selective index of the extracts.

Chapter 5

Cytotoxic evaluation of the plant extracts against Vero African green monkey kidney cells

5.1 Introduction

It is estimated that over 122 drugs have been discovered from 94 plant species through ethnobotanical leads. One out of four drugs found in pharmacies contain an active ingredient obtained from a plant (Booth *et al.*, 2011). Such drugs include arecoline, colchicine and aescin isolated from *Areca catechu* L.*, Colchicum autumnale* L. and *Aesculus hippocastanum* L. respectively (Fabricant and Farnsworth, 2001). Plants also play a vital role in the economy, as it is estimated that over $18 billion (US) is made in the international consumer market for medicinal herbs and botanicals (Booth *et al.*, 2011). In South Africa approximately 75% of the population use medicinal plants with a value of about R2.9-4 billion per annum, in Bushbuckridge alone over 500 tonnes of traditional medicines are traded (De Bruin, 2015; Chauke *et al*., 2015).

Since medicinal plants are from a natural origin and have been used for centuries to treat diseases there is an assumption that plant remedies are non-toxic and safe to use (Chen *et al*., 2011; Fennell *et al*., 2004). However, scientific studies have reported many plants used in traditional remedies as toxic, mutagenic and carcinogenic (Fennell *et al*., 2004). Toxicity of medicinal plants has adverse effects ranging from mild inflammation to organ failure and death in both humans and animals. Toxicity from herbal remedies may affect the liver, heart, kidney, nervous system and skin (Chen *et al*., 2011).

Poisoning from plant remedies can be caused by various factors including errors during preparation, inappropriate administration, and consumption of high dosages and misidentification of a particular plant (Mamabolo *et al*., 2018; Fennell *et al*., 2004). The consumption of medicinal plants simultaneously with conventional drugs leads to the development of dangerous herb-drug interaction which may cause altered drug response and toxicity especially if the drugs have little difference between toxic and therapeutic doses, for example warfarin and digoxin (Chen *et al*., 2011). Similarly to synthetic drugs, bioactive compounds in medicinal plants can also undergo Phase I and Phase II reactions to form non-toxic metabolites which are excreted through urine and faeces. However, the synthesis of reactive and potentially toxic metabolites is feasible and has significant toxicological implications (Chen *et al*., 2011). Furthermore, there is no regulatory system for prescription

and use of traditional medicine in South Africa and therefore, it is very important to acquire information on the toxicity and efficacy of the plants used to treat animal and human ailments.

The toxicity level of plant extracts can be determined using various assays, including those using lower organisms, biochemical assays, cell cultures and isolated organs. Cell culture assays are preferred as they are common, rapid, inexpensive and do not have ethical implications (Fernandez *et al*., 2005). In terms of *in vitro* cell culture systems, a tested plant extract is considered cytotoxic if it interferes with the attachment of cells, significantly alters morphology, adversely affects the rate of cell growth, or causes cells to die (McGaw *et al.*, 2014). There is a relationship between toxic concentrations determined *in vitro* to those studied *in vivo*. This is because the mechanism of action of toxicity is associated with basic biochemical processes, which are common to all cells. These basic processes include protein synthesis (mitochondrial) and cell membrane integrity (McGaw *et al.*, 2014).

Since there is an increasing emphasis on integrating traditional medicine into primary health care it is important to study the toxicity potential and efficacy of traditional remedies as there is limited information about their relative safety (Fennell *et al*., 2004). It is important to evaluate the intrinsic ability of plant extracts or compounds to cause cell death as a result of damage to several cellular functions, thus *in vitro* cytotoxicity is essential as a first step in such an evaluation (Fennell *et al*., 2004). Toxicology bioassays are necessary in order to scientifically recommend the safe use of non-toxic plants, thus in this study the selected medicinal plants (Table 4) were selected due to their antimicrobial activity. The aim of this study was to determine the potential risk of crude extracts by evaluating the cytotoxicity against Vero African green monkey kidney cells.

5.2 Materials and Methods

5.2.1 Plant preparation

Plant extracts were prepared as described in section 4.2.1. The dried plant extract was reconstituted with water and acetone at a concentration of 100 mg/ml. Serial dilutions were made, and concentrations starting from 1.0 mg/ml to 0.0075 mg/ml were tested in the study.

5.2.2 The 3-(4,5-dimethyltetrazolium bromide) (MTT) reduction assay

Plant extracts were tested against Vero African green monkey kidney cells (ATCC® CCL-81™) using the 3-(4,5-dimethyltetrazolium bromide) (MTT) reduction assay described by Mosmann (1983) with slight modifications. Cells were seeded at a density of 1 x 10^5 cells/ml (100 µl) in 96-well microtitre plates and incubated at 37ºC in a 5% CO_2 humidified incubator for 24 h to attach. After the incubation period, 100 µl of each extract were added to the wells

containing cells. Doxorubicin was used as a positive control. Negative controls with equivalent concentrations of the solvents were also included, and the plates were further incubated for 48 h in a CO_2 incubator. Thereafter, the medium in each well was aspirated from the cells which were then washed with PBS. Finally fresh medium (200 µl) was added to all the wells, and 30 µl of MTT (5 mg/ml in PBS) was added to each well and the microtitre plates were incubated at 37ºC for 4 h. Following 4 h incubation, the medium was aspirated from the wells, and 50 µl of DMSO added to solubilize the formed formazan crystals. The absorbance was measured on a BioTek Synergy microplate reader at 570 nm and reference wavelength of 630 nm. The IC_{50} values were calculated as the concentration of plant extract resulting in a 50% reduction of absorbance compared to untreated cells. The relative safety of each extract can be assessed using the selectivity index, which is calculated as follows:

Selective index (SI)= IC_{50}/MIC

Where IC_{50} = Inhibitory concentration that inhibits 50% of the Vero cells, MIC = Minimum Inhibitory Concentration against *E. faecalis, S. aureus E. coli* and *P. aeruginosa.* The MIC results used to calculate the selective index were obtained from Chapter 4 of the present study.

5.3 Results and Discussion

Nine plant extracts were tested in this study (Table 5.1) for cytotoxicity. According to Efferth and Kuete (2010) a crude extract is considered to have *in vitro* cytotoxicity if the IC_{50} after incubation for 48 h is less than 20 µg/ml (0.02 mg/ml) after a 48 h incubation period.

A. *marlothii* (infusion) was the least cytotoxic with the highest IC_{50} value of 0.205 mg/ml. *S*. *brachypetala* (aqueous) also had low toxicity with IC_{50} value of 0.105 mg/ml. *E. obliqua* acetone and water (decoction) extracts were the most toxic plant extracts with IC_{50} values of 0.015 and 0.021 mg/ml respectively. *S. brachypetala* water extracts had lower toxicity with IC_{50} value ranging from 0.090 mg/ml to 0.105 mg/ml compared to the acetone extract with an IC_{50} of 0.044 mg/ml. Out of all the tested crude extracts water, extracts had lower toxicity than organic solvent (acetone) extracts.

Table 5.1: The cytotoxicity (presented as Inhibitory Concentration 50%, IC_{50}) and selectivity index results of extracts from plants used by the Mnisi community for animal health care

Plant extracts	Cytotoxicity	Selective Index								
	IC50 (mg/ml)	*Ec*	*Pa*	*Ef*	*Sa*	*Ca*	*Cn*	*Af*	*Mt*	*Mb*
Aloe marlothii										
Acetone	0.053± 0.025	0.252	0.408	0.408	0.279	0.042	0.065	0.044	0.084	0.044
Infusion	0.205 ± 0.037	0.301	0.236	0.477	0.281	0.082	0.126	0.099		
Elephantorrhiza obliqua										
Acetone	0.015 ± 0.013	0.056	0.161	0.107	0.024	0.006	0.007	0.024	0.047	0.047
Decoction	0.021 ± 0.010	0.020	0.062	0.095	0.017	0.008	0.013	0.700	0.066	0.131
Aqueous	0.047 ± 0.016	0.075	0.127	0.147	0.075	0.019	0.023	2.350		
Infusion	0.038 ± 0.020	0.037	0.086	0.173	0.045	0.015	0.018	0.950	0.238	0.475
Schotia brachypetala										
Acetone	0.044 ± 0.008	0.069	0.136	0.396	0.035	0.018	0.027	0.070	1.245	1.089
Decoction	0.090 ± 0.027	0.043	0.036	0.375	0.036	0.036	0.063	0.072	-	-
Aqueous	0.105 ± 0.156	0.101	0.063	0.167	0.084	0.167	0.167	0.128	5.25	2.625
Doxorubicin	0.005 ± 0.009									

hydrochloride		

Microbial strains: *Escherichia coli (Ec), Enterococcus faecalis (Ef), Pseudomonas aeruginosa (Pa), Staphylococcus aureus (Sa), Candida albicans (Ca), Cryptococcus neoformans (Cn)* and *Aspergillus fumigatus(Af), Mycobacterium tuberculosis (Mt)* and *Mycobacterium bovis (Mb).*

Medicinal plant extracts proposed for use in clinical applications must not have a significant effect on the host cell or interfere with its normal physiological pathway. The extracts should be selectively toxic to the targeted organism or interfere directly with a specific reaction pathway, and this must be done without disturbing the host cell or the normal physiological pathway (Kuete *et al*., 2011). The principle of the MTT assay is based on the reduction of a soluble tetrazolium salt by mitochondrial dehydrogenase activity of viable cells into a soluble purple coloured formazan compound that is easily measured using a spectrophotometer after it has dissolved (Ariffin *et al*., 2009). The IC_{50} is used as a parameter for cytotoxicity. According to the American National Cancer Institute (NCI), a plant crude extract is considered cytotoxic if it shows an IC_{50} value of 30 µg/ml (0.03mglml) or below following incubation between 48 and 72 hours (Kudumela and Masoko, 2018; Sudha and Masilamani, 2012; Mena-Rejon *et al.*, 2009). Therefore, analysis of the results for the present study were interpreted as follows: highly toxic for IC_{50} values < 0.03 mg/ml, moderately toxic 0.03 mg/ml < IC_{50} ≤ 0.1 mg/ml and non-toxic IC_{50} > 0.1 mg/ml.

Cellular viability and proliferation are considered to be the most fundamental characteristics of healthy growing cells. The increase in cell viability indicates cell proliferation, while decrease in cell viability indicates cell death which can be as a result of toxic effect of the tested extracts or substandard culture conditions (Sudha and Masilamani, 2012). Out of the nine plant extracts tested only one had an IC_{50} value below 20 µg/ml. The most cytotoxic extracts were prepared from *E. obliqua (*acetone and decoction). It must be taken into consideration that there are limitations to be considered when comparing the results between *in vitro* and *in vivo* studies. The limitations are influenced by different conditions within the two systems (McGaw *et al*., 2014). Toxicity *in vivo* may result from tissue response possibly caused by an inflammatory reaction, kidney failure, or even systemic response. Various levels of activity may be reflected by different organs depending on the metabolic rate and other mechanisms (McGaw *et al*., 2014). However, toxic responses *in vitro* are evaluated by the changes in the survival or metabolism of cells which are closely related to tissues or systemic toxicity (Kudumela and Masoko, 2018; McGaw *et al*., 2014).

Although some of the extracts showed some degree of cytotoxicity *in vitro* against the Vero cells at concentrations tested, according to an extensive literature search, there are no toxicity reports that have been made concerning *E. obliqua*. However, the plants might be less toxic *in vivo* as cellular toxicity varies from whole animal toxicity due to different biochemical interactions in the gut. It is important to note that although there have been cytotoxic studies done on various *Elephantorrhiza* species no information has yet been documented on the toxicity of *E. obliqua*. It was noteworthy to observe that *A. marlothii* infusion extract had the highest IC_{50} value (0.205 mg/ml) out of all the extracts tested against

the Vero cells which means it was relatively non-cytotoxic. Out of all the tested plant extracts *S. brachypetala* water extract was the second least cytotoxic extract. Similar results were found by McGaw *et al.* (2007) where *S*. *brachypetala* (bark) water extract was non-toxic against brine shrimp larvae.

The results obtained in this study are in agreement with the results reported by Fouche *et al*. (2008) where it was found that out of 7 500 plants screened for anticancer activity all aqueous extracts were non-toxic against the tested cell lines compared to organic solvent extracts. This may be because water as a solvent is unable to extract large quantities of cytotoxic constituents from the plant. The toxicity in organic solvent extracts may be due to the high quantities of cytotoxic components extracted during plant extraction. According to Kudumela and Masoko (2018) and respondents in this study, traditional remedies have no side effects and are less toxic. However, extracts such as *E. obliqua* infusion with low IC_{50} values reflecting cytotoxicity should be carefully monitored although there are no reports of toxicity.

The selectivity index was used to relate the cytotoxicity and antimicrobial activity of the plant extracts. The biological activity of the plant extracts is generally considered not to be due to *in vitro* cytotoxicity if the selectivity index is ≥ 10 (Kudumela *et al*., 2018; McGaw *et al*., 2014). The plant extracts in the present study had low selectivity index values ranging from 0.017 to 5.52 (Table 5.1). All plant extracts had SI values below 10 but it is promising that many had SI values above 1, which means that their biological activity was higher than their cellular toxicity. *S. brachypetala* aqueous extract had the highest SI values against the mycobacterial pathogens with SI values of 5.25 and 2.6. Sl values of all extracts were low against fungal strains, and similar results were reported by Masevhe (2015), where water, acetone and methanol extracts *of S. brachypetala* leaves had low SI values (SI<10) when tested against *Candida albicans* and *Cryptococus neoformans* fungal strains. However it is also important to note that cytotoxicity *in vitro* is not always found *in vivo,* because some compounds may be subjected to metabolic transformation within the biological system resulting in less toxic products (Kudumela *et al*., 2018). However the reverse may also be true where more toxic chemicals are formed as a result of metabolic activity.

5.4 Conclusion

Traditionally prepared aqueous extracts were found to be less toxic than organic solvent extracts against Vero cells. Some extracts had good selectivity index values indicating that activity was much better than cytotoxicity, but no extract had an SI value above 10 which is often considered to be a useful target for identifying attractive plant extracts for further scientific investigation. There might be risks associated with consumption of some of the remedies because according to the SI results there is a possibility that the antimicrobial activity of the remedies might be due to toxicity. The dose used is extremely important as are other factors such as variation in chemical composition of plant constituents in different areas and at different times of the year. Standardisation of extracts is recommended if they are to be produced commercially. It is essential to conduct *in vivo* studies to conclusively evaluate the safety, as well as efficacy of the plant extracts, as toxicity *in vitro* might not translate to toxicity *in vivo*.

Chapter 6

General conclusions

There are many reports on traditional remedies used to treat humans, however there are fewer reports on traditional remedies used for animal health care. Thus the aim of this study was to document and investigate plant species that are used as ethnoveterinary medicine by the Mnisi community at Bushbuckridge in Mpumalanga and to evaluate their antimicrobial activity. In order to achieve this aim several objectives were formulated. The results obtained in addressing the various objectives and their significance are discussed below.

6.1 Conduct a survey to obtain information on plants used in EVM, their methods of preparation and administration

In an endeavour to obtain knowledge about the plant remedies used to treat animal related ailments, a survey was carried out where 50 individuals were interviewed. It was found that the respondents of the Mnisi community used eleven plant species belonging to seven plant families for EVM. Fresh plants collected from the wild were used to prepare the remedies as decoctions, infusions, pastes and extracted sap. Water was the only solvent used to prepare the remedies and the remedies were administered orally or applied directly on the infected area (wound). The remedies were used to treat various diseases including diarrhoea, black quarter, gall related diseases, foot and mouth disease, eye infections, swollen stomach, sudden weight loss and wounds. Knowledge of traditional remedies is relayed orally therefore the information documented in this study will be a permanent record which can be used in the future for ethnopharmacological studies. Plant species such as *A. marlothii*, *C. quadrangularis and S.brachypetala* recorded in this study are also used by other ethnic groups from different regions such as the Tswana tribe from Madikwe, Venda tribe from Vhembe region and the Tsonga from Giyani. (Chauke *et al*., 2015; Luseba and Tshisikhawe, 2013; Luseba and Van der Merwe, 2006; Van der Merwe *et al*., 2001). Some of the plants are used for different disease in other areas while some are used for similar diseases. There is no documentation of *Elephantorrhiza obliqua* amongst other ethnic groups; however they use *E. elephantina* to treat similar diseases such as diarrhoea and black quarter which are treated with *E. obliqua* in the Mnisi community. The Fabaceae family is one of the dominant

families used by various ethnic groups such as Vendas, Tswanas and Tsonga (Luseba and Tshisikhawe, 2013; Van der Merwe *et al*., 2001). It is still important to investigate EVM remedies used by other ethnic groups in various regions as they will provide more EVM knowledge.

6.2 Plant collection and identification

Plant material was collected in various regions of the Mnisi community under the guidance of the respondents and the environmental monitor. Photographs of the plants were taken along with the precise geographical coordinates of the collection site. Plant specimens were labelled, pressed and identified at the HGWJ Schweickerdt Herbarium, University of Pretoria. Plants which were frequently used for prevalent diseases (diarrhoea and black quarter) and those less investigated in literature were selected and analysed for antimicrobial activity.

6.3 Perform laboratory assays to test and compare the activity of the plants prepared in the traditional way and using organic solvents against selected bacterial, fungal and mycobacterial pathogens

Nine plant extracts from three plant species selected were prepared traditionally as described by the respondents using water as a solvent (decoctions, infusions and aqueous laboratory-prepared) and also an organic solvent (acetone) was used as a comparison. The biological activity of the extracts was tested against various microbial pathogens including bacterial strains *(E. coli, S. aureus, E. faecalis* and *P. aeruginosa*), fungal strains (C*. albicans, C. neoformans* and *A. fumigatus*) and mycobacterial strains (*M. tuberculosis* and *M. bovis*). The two-fold serial microdilution method was used to determine the MIC values of the extracts. *Elephantorrhiza obliqua* acetone extract had the best antibacterial activity with an MIC value of 0.09 mg/ml against *P. aeruginosa*, while *E. obliqua* water (decoction) extracts had the highest antifungal activity with MIC values of 0.02 to 0.04 mg/ml against *Aspergillus fumigatus*. *Schotia brachypetala* aqueous extract had the highest antimycobacterial activity (MIC = 0.02 mg/ml).

S. brachypetala acetone extracts had highest biofilm inhibition of 113% at T0 and 135% at T24, while *E. obliqua* acetone extracts inhibited biofilms at 64% at T0 and 83% at T24. The water extracts lacked activity against both planktonic and biofilm forms of bacteria and therefore may have a different mechanism of action against infectious diseases. Although water extracts had little activity against bacterial strains it was encouraging to note that water

extracts were the most active against fungal and mycobacterial pathogens compared to the organic solvent acetone. The results are exciting because water is the only solvent freely used in rural communities such as the Mnisi community as organic solvents are not available. It is also interesting to observe that some of the plant extracts had better activity than the positive controls thus the extracts should be further investigated and responsible compounds should be isolated and evaluated as this may contribute to drug development or standardisation of active extracts or fractions.

6.4 Test the plant extracts for cytotoxicity

As a preliminary investigation of *in vitro* safety of the plant extracts, cytotoxicity was evaluated against Vero monkey kidney cells using the MTT assay. *Aloe marlothii* (infusion) had the lowest cytotoxicity and *Schotia brachypetala* (aqueous) also had low toxicity to the cells. Although the *E. obliqua* acetone extract and decoction had the highest antibacterial and antifungal activity, they were both cytotoxic and had SI values below one against all strains. *S. brachypetala* acetone and aqueous extracts had the highest antibiofilm and antimycobacterial activity and *S. brachypetala* aqueous extract was non-cytotoxic while *S. brachypetala* acetone extract had low cytotoxicity. The selectivity index of the extracts ranged and lower SI values suggest that those extracts may be more suitable for external use and therefore they should be used with caution when treating various ailments and taken internally.

It is important to conduct *in vivo* studies in order to conclusively evaluate the safety as well as efficacy of the plant extracts as toxicity *in vitro* might not translate to toxicity *in vivo*. In this section valuable information was obtained that will contribute to the knowledge of safety of traditional medicine.

6.5 Conclusion and recommendations for future work

The people of the Mnisi community have been using traditional medicine for many years for both animal and human health care. However there are no previous ethnoveterinary records documented from the area. Older people in the community have an abundance of traditional medicinal knowledge, especially concerning remedies used to treat domestic animals such as cattle. However the youth have little to no knowledge about traditional medicine which may be as a result of them migrating to urban areas to seek employment opportunities, where ethnoveterinary knowledge is of little practical value. Thus the chain of transfer of

ethnoveterinary knowledge from generation to generation is broken as a result of the modern circumstances of young people. In this study a survey was conducted on plants used for ethnoveterinary medicine in the Mnisi community, Mpumalanga. The survey was conducted using a questionnaire and older individuals were found to have more EVM knowledge than the youth. Fifty people were interviewed at five different dipping tanks, and the majority were male. Only 31 people had EVM knowledge and eleven plant species from seven families were identified as being used in EVM. Laboratory investigations were conducted towards providing scientific support for the use of the selected plants in ethnoveterinary medicine. The efficacy and *in vitro* safety of the plant extracts was investigated, comparing laboratory prepared extracts with decoctions and infusions prepared in the traditional manner. It was found that traditionally prepared remedies had better antifungal and antimycobacterial activity while organic solvent extracts had better antibacterial activity. Notable information of the biological activity of *Elephantorrhiza obliqua* was recorded.The study showed the importance of studying traditional medicine in search of active plant extracts that may lead to the development of low cost drugs for combating various veterinary-related ailments.

For future work, it is necessary to give a feedback presentation to the community to provide them with the results of the work conducted in this project. Reports written in the local language are essential so that local people can understand the compiled information easily. Elders may pass the written, documented information to their younger generation as it is difficult to gather all the youth at the same time.

In order to obtain more knowledge in the long term the following research goals should be adopted: More comprehensive studies should be carried out in different regions of southern Africa to develop a powerful database on ethnoveterinary medicinal plants and ethnoveterinary practices. Patterns and trends of use can be analysed to determine useful leads for in depth scientific investigation.

The biological/pharmacological properties of the plant remedies should be studied more widely. The plants that are safe and effective in laboratory studies and then in animal trials should be considered for the development of cheaper herbal remedies that can be afforded by poor people in rural communities. Such remedies would be available all year round and not just seasonally when plant material is available. Standardisation of extracts could also be valuable in quality control and in ensuring that correct doses and sufficient active substances are present in the remedies. Cultivation and sustainability of plant material as a source of active ingredients should also be addressed when developing a herbal remedy.

Chapter 7

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Appendix 1

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

9/11/2017

Approval Certificate
New Application

Ethics Reference No: 376/2017

Title: Plants used in ethnoveterinary medicine to treat prevalent livestock diseases in the Mnisi community, Mpumalanga Province, South Africa

Dear Mr Edward ET Khunoana

The **New Application** as supported by documents specified in your cover letter dated 6/11/2017 for your research received on the 6/11/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 8/11/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**376/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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Appendix 2

Questionnaire

Section A [Personal details are only requested for follow-up purposes and will be kept anonymous]

1. What is your name?
2. How old are you?
3. What is your occupation?
4. What is your level of education?
5. How long have you stayed in this area?
6. Do you know anything about ethnoveterinary medicine (if not it will be explained to the interviewee)?
7. What are your views on ethnoveterinary medicine?

Section B

1. Which diseases are affecting your livestock?
2. Which animals are affected?
3. What are the symptoms of the disease?
4. Which plants are used to cure the mentioned diseases?
5. How are the plants prepared?
6. Which part of the plant is used?
7. How is the plant administered to the animal?
8. What unit of measurement is used to measure the medicine?
9. What is the maximum dosage administered to the animals?

Section C

1. Where are the plants collected?
2. Are those plants that you use for treatment available in your area?
3. How are the plants stored after collection?
4. At what growth level can the plants be used?
5. At what stage of infection can the plants be used?
6. What are the side effects of the plants on the animal?
7. How long is the animal treated?
8. Has any other part of the plant been used before?
9. Have you tried preparing the plants in a different manner (if yes what were the results)?
10. How is information passed amongst ethnopractitioners?

Section D

1. How effective is your treatment?
2. Which medicinal plants have you used the most for treatment?
3. When do these plants grow (season)?
4. What do you do to ensure that there are plants for you to harvest in the future?
5. Do you move to new areas to buy medicinal plants?
6. Do you use cultivated plants for the treatment?
7. How long can you keep medicinal plants before they lose their healing properties?