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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Highlights

• An ethnoveterinary survey in Mnisi, Bushbuckridge, Mpumalanga documented use of 11 plant species.

• Selected plants were extracted using traditional methods as well as acetone and distilled water.

• Extracts were evaluated for antimicrobial, antibiofilm and cytotoxic activities.

• Traditionally prepared remedies were more active against fungi and mycobacteria and less toxic than organic extracts.

• Only two plant extracts were relatively cytotoxic to Vero cells.

Abstract

Ethnoveterinary medicine (EVM), although not documented well, still serves as an alternative or complementary medication for curing or preventing bacterial, fungal and helminth diseases, as well as other maladies such as ticks and tick-borne diseases in South Africa. The aim of this study was to document plant species used as EVM by the Mnisi community at Bushbuckridge in the province of Mpumalanga, and to evaluate their antimicrobial, antibiofilm and cytotoxic activities**.** The survey was carried out for two weeks from the end of January to the beginning of February 2018 at the local dipping tanks following the Rapid Rural Appraisal (RRA) approach. A total of 50 individuals were interviewed: 82% were local small scale farmers, 10% were herdsmen, 6% herbalists and 2% animal health technicians. Three plant species were selected for bioassays based on their frequency index and lack of information on their bioactivity in the literature. Traditional methods were used for plant extraction using water as described by the respondents. Acetone was used as an organic solvent to compare traditional and organic solvent methods of extract preparation. The extracts were tested for their antibacterial, antibiofilm, antifungal, and cytotoxic properties.

Eleven plant species belonging to seven families were reported by the farmers for EVM use, and fresh plants from the wild were commonly used to prepare the remedies as decoctions, infusions, pastes and extracted sap. *Elephantorrhiza obliqua* acetone extract had the best antibacterial activity with a minimum inhibitory concentration (MIC) value of 0.09 mg/ml against *Pseudomonas aeruginosa*, while *E. obliqua* water extracts had the best antifungal activity with MIC values of 0.02 to 0.04 mg/ml against *Aspergillus fumigatus. Schotia brachypetala* acetone extracts inhibited *Enterococcus faecalis* biofilms by 113% and 135% at zero and 24 h 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. Traditionally prepared remedies were generally more active against fungi and mycobacteria and less toxic than the organic solvent extracts. However, *in vivo* studies are 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.

Keywords: antibacterial, antibiofilm, antifungal, antimycobacterial, cytotoxicity, ethnoveterinary medicine, Mpumalanga

1. **Introduction**

Worldwide, 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). In South Africa, farmers of different ethnic groups, including Tswana, Tsonga, Xhosa and Zulu, 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 (McGaw and Eloff, 2008). The application of these traditional practices constitutes ethnoveterinary medicine (EVM). 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). EVM is used mostly in developing countries like South Africa. In these countries 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).

In South Africa traditional knowledge is commonly passed on from one generation to the next in oral format (McGaw and Eloff, 2008). 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. 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. Thus generalization of results from one area to another is not possible. (Ramadwa, 2010; Maphosa and Masika, 2010; Luseba and Van der Merwe, 2006)

Modern pharmaceuticals are gradually failing to prevent or cure infectious diseases, and this is a result of different microorganisms adapting to antimicrobial agents by developing resistance to them. Antimicrobial 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 (Van Vuuren and Holl, 2017). Traditional medicine used for human treatment developed parallel with that for animal treatment, yet human treatment has more documentation compared to animal treatment (Maphosa and Masika, 2010).

In South Africa, relatively few ethnoveterinary surveys have been conducted and even fewer remedies have been tested for ethnoveterinary efficacy and safety (McGaw and Eloff, 2008). The migration of indigenous people to urban areas has resulted in the loss of indigenous traditions in favour of Western-derived traditions and since traditional medicine knowledge is passed on from generation to generation orally there is a concern that the information may be lost or inadequate information may be passed on to future generations (Maphosa and Masika, 2010; McGaw and Eloff, 2008; Luseba and Van der Merwe, 2006; Van der Merwe *et al*., 2001). 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 obtaining 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) with the aims of documenting EVM practices and validating them scientifically.

2. Materials and methods

2.1 Study area

The study was carried out in the Mnisi community **(Figures 1 and 2)** in Bushbuckridge, Mpumalanga, 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 and spread of different diseases (De Bruin, 2015).

Figure 1: Map of the study area within the Bushbuckridge local municipal area (De Bruin, 2015)

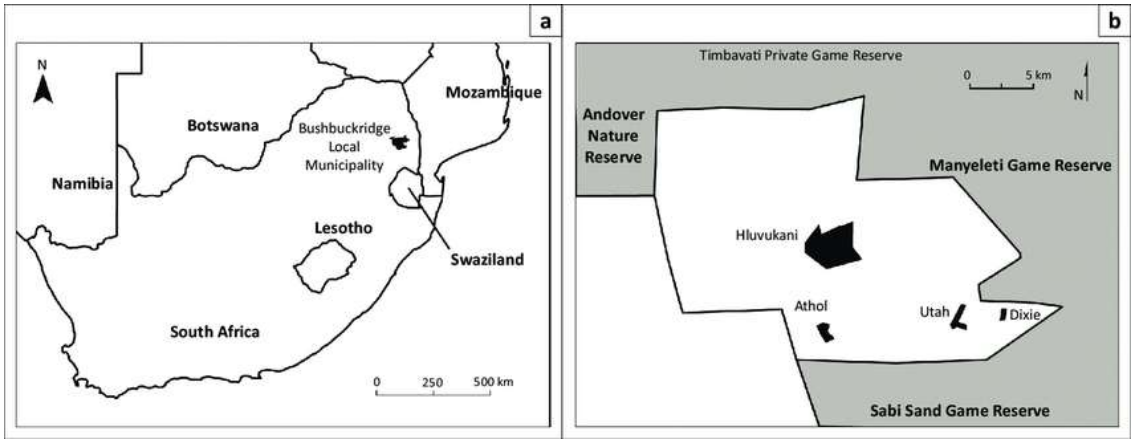

Figure 2: Map of the study area within the country

2.2 Survey

The survey was conducted following methods described by Weckerle et al. (2018) and Heinrich et al. (2017) comprising the Rapid Rural Appraisal (RRA) approach. A meeting was held with the traditional chief and the dip tank committees of the local villages within the 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 (questionnaires), general conversations, observations and field walks. The survey commenced from the end of January to the beginning of February 2018 for two weeks. The interviewed individuals comprised local small scale farmers (41), animal health technicians (1), herdsmen (5) and herbalists (3). Ethics permission was granted by the Faculty of Health Sciences, University of Pretoria (Ethics Reference No 376/2017).

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 survey (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).

2.3 Plant collection and extraction

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. 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 identified at the HGWJ Schweickerdt Herbarium, University of Pretoria, where voucher specimens were deposited. Traditional methods were used for plant extraction as described or prepared by the respondents. Acetone was also used as an extractant in order to compare the traditional and organic solvent methods of extract preparation. Three plant species that were frequently cited and not fully 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* Burtt Davy, bark of *Schotia brachypetala* Sond. and leaves of *Aloe marlothii* A.Berger. 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 dried 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 it 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 same 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 or water).

2.4 Antimicrobial activity of crude extracts against bacterial, fungal and *Mycobacterium* strains

2.4.1 Microbial strains

The National Committee for Clinical Laboratory Standards (NCCLS, now CLSI or Clinical and Laboratory Standards Institute) recommend that Gram-positive *Enterococcus faecalis* (American Type Culture Collection (ATCC) 29212) and *Staphylococcus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) should be used for routine antibacterial screening, thus in this study these bacterial strains were selected (McGaw and Eloff, 2010). The bacterial strains were maintained on Mueller-Hinton (MH) agar at 37°C, cultured in MH broth and incubated for 16 hours at 37°C before screening.

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 a mould species *Aspergillus fumigatus*. 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.

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

2.4.2 Antibacterial assay to determine the minimum inhibitory concentration (MIC) values

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 bacterial species. Two-fold serial dilutions were prepared in 96-well microtitre plates as follows: sterile distilled water (100 µl) was pipetted in each well and 100 µl of a 10 mg/ml plant extract were added to the first three wells and serially diluted down them, and the last rows were discarded to ensure that all wells contained the same volume. Exactly 100 µl of overnight cultured bacteria was added in each well. Gentamicin was used as a positive control whereas acetone and sterile distilled water were used as negative controls (Eloff, 1998b). The plates were incubated for 18 h at 37°C in a humidified atmosphere. Following the 18 h incubation period, 40 µl of *p*-iodonitrotetrazolium (INT) (0.2 mg/ml) dissolved in water were added to each well. The MIC value 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.

2.4.3 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 h of bacterial incubation). A 100 µl aliquot of standardized concentration of *E. faecalis* culture ($1x10^6$ CFU/ml) was added into wells of microtitre plates and incubated at 37°C for 0 and 24 h without shaking. After the selected incubation periods, extracts (100 µl of 1 mg/ml concentrations) were added.

Gentamicin (1 mg/ml initial concentration) 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 was determined and percentage inhibition was calculated following the equation below (Sandasi *et al.*, 2011).

$$\text{Percentage inhibition} = \{\frac{ODNegative\ control - ODExperimental}{ODNegative\ control}\} * 100$$

2.4.4 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 MIC values for plant extracts against fungal strains. All wells of sterile 96-well microplates were filled with 100 µl of sterile distilled water. Exactly 100 µl of a 10 mg/ml plant extract were added in each of the first wells and serially diluted two-fold down the wells. Fungal cultures ($1x10^5$ CFU/ml) grown in SD broth were added to each well (100 µl). Amphotericin B was used as a positive control. As an indicator of growth 40 µl of 0.2 mg/ml of INT dissolved in sterile distilled water were added to all the wells. The microtitre plates were incubated for 24 h at 37°C and 100% relative humidity. The MIC value

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.

2.4.5 Antimycobacterial assay to determine the minimum inhibitory concentration (MIC) values

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. Colonies of 1 month culture of each mycobacterial strain were transferred into 3 ml of oleic acid albumin dextrose catalase (OADC) supplemented Middlebrook 7H9 broth and homogenized by vortexing, allowing larger particles to settle. Supplemented Middlebrook 7H9 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 microdilution 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 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 INT solution was added to each well to determine the MIC values. 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.

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

Plant extracts (concentrations from 1.0 mg/ml to 0.0075 mg/ml) 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 concentration of the 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 5% CO_2 incubator. Thereafter, the medium in each well was aspirated from the cells which were then washed with phosphate buffered saline (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 microtitreplates were incubated at 37ºC for 4 h. Following 4 h incubation, the medium was aspirated from the wells, and 50 µl of DMSO were 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)= $\frac{LC50}{MIC}$

Where IC_{50} = Inhibitory concentration that inhibits 50% of the Vero cells, MIC = Minimum Inhibitory Concentration against bacterial strains tested.

3 Results and discussion

3.1 Survey

3.1.1 Informants

During the survey 50 people were interviewed at five different dipping tanks in the Mnisi community. The interviewees consisted mostly of farmers (82%) and some herdsmen (10%). Out of the 50 interviewees, 10 were female farmers. Most (31) individuals had knowledge about EVM while the remaining 18 individuals had no knowledge, and thus the information was acquired from 31 informants. 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).

Similar results were obtained in the surveys done by Luseba and Van der Merwe (2006) and Maphosa and Masika (2010), where males above the age of 40 were the most knowledgeable age group when coming to 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).

3.1.2 Diseases affecting the community livestock

The Mnisi community livestock is continually at risk of contracting different diseases due to its proximity to wildlife. At the time of the study, the community was 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 and conditions 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 results 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. 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.

3.1.3 Plants used as ethnoveterinary medicine

During the survey, eleven plant species belonging to seven families were reported (Table 1). The Fabaceae was the most represented family with five plant species. This family also contained two of the top three most frequently mentioned plant species, and they included *Elephantorrhiza obliqua* Burtt Davy which was mentioned by 48% of the respondents for the same disease and *Senna italica* Mill. with 19%. *Aloe marlothii* A.Berger from the Asphodelaceae family was also part of the top three with 39% of people mentioning it for ethnoveterinary use. The Fabaceae family is one of the most used plant families in EVM amongst different ethnic groups in South Africa which is not surprising as it is the second largest family of plants in this country 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 1: Plants used in ethnoveterinary medicine with 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 orally from one generation to the next and 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.

3.1.4 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 root 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 people (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 preferred to use fresh plant material collected from the wild. Plants were not collected for storage or planted at private homes but only collected when they were needed which was supported by earlier reports of Luseba and Tshisikhawe (2013).

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.

3.1.5 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.

3.2 Minimum inhibitory concentration against microbial strains

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 two pathogenic mycobacterial species and the MIC values are presented in Table 2. 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 had MIC values against a microbial test organism below 100 µg/ml (0.1 mg/ml) (Eloff 2004; Rios and Recio, 2005).

Table 2: Antimicrobial activity of nine extracts from three plant species against bacterial, fungal and mycobacterial strains

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*)*, -=not tested

3.3 Biofilm activity

The biofilm inhibitory activity (BIA) of the crude extracts against pathogenic *Enterococcus faecalis* is presented in Figure 3. Out of all the tested bacterial strains the plant extracts had the best 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.

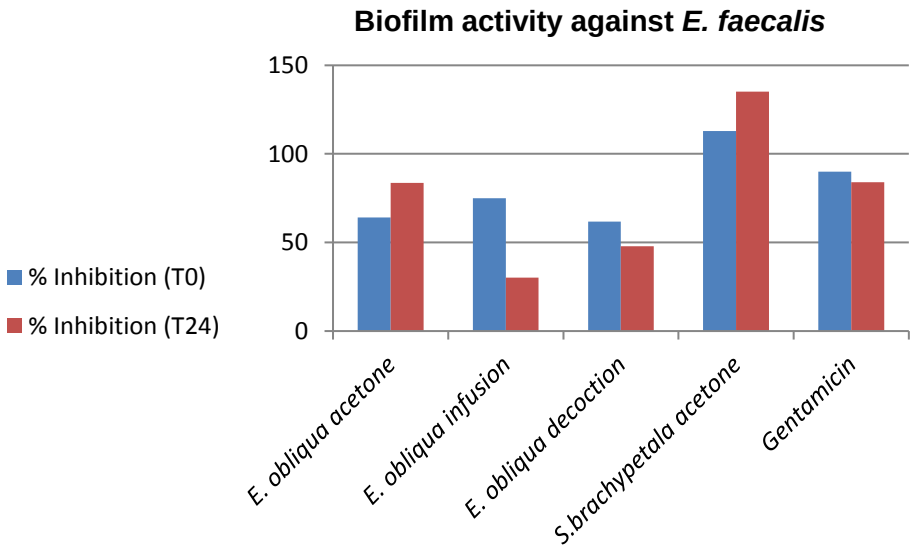

Figure 3: Inhibitory percentage of four plant extracts against *E. faecalis* biofilm at 0 hour and 24 h.

All the tested plant extracts had MIC values ranging from 0.02 to 2.50 mg/ml against 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 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 were overall the most active with the acetone extract having the best antibacterial activity with an MIC value of 0.09 mg/ml against *P. aeruginosa*, while water extracts of the same plant 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 antibacterial 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 2).

It was 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 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 2 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 inhibited biofilm by 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 prevent the biofilm from developing into a matured stage.

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

Nine plant extracts were tested in this study (Table 3) for cytotoxicity. Out of all the tested crude extracts, water extracts had lower toxicity than organic solvent (acetone) extracts.

Table 3: 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	Selectivity Index								
	IC_{50} (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 hydrochloride	0.005 ± 0.009									

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 (0.03 mg/ml) or below following incubation between 48 and 72 h (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 < $IC_{50} \leq 0.1$ 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 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 done 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 less 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 and Masoko, 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 3). 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. SI 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 and Masoko, 2018), and the reverse may also be true where more toxic chemicals are formed as a result of metabolic activity.

4. **Conclusion**

This study provides evidence that the use of plant remedies to treat livestock diseases is still common amongst the people of the Mnisi community in the Bushbuckridge municipality. 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. 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. Although traditionally prepared extracts did not yield highly active antibacterial properties, the extracts

showed significant antifungal and antimycobacterial activities and were less toxic towards Vero cells. 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 currently used as one of the main defence pharmaceuticals against fungal 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* should be subjected to further studies including isolation of antimicrobial compounds. Some extracts had good SI values indicating that activity was much better than cytotoxicity, but less than 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. Therefore, 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*.

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