University of Venda

An inventory and pharmacological evaluation of medicinal plants used as anti-diabetes and anti-arthritis in Vhembe District Municipality, Limpopo Province, RSA

By

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Abstract

Diabetes and arthritis are the most common chronic diseases. Arthritis is the leading cause of global disability and diabetes has become a major health problem which is increasing rapidly. The purpose of the study was to document medicinal plants that are used to treat and manage diabetes and arthritis by traditional medicinal practitioners around the Vhembe District Municipality as well as to evaluate their *in vitro* efficacy. Traditional practitioners were interviewed using semi-structured questionnaires. Seventeen plant species belonging to fourteen different families were found to be used in the treatment of diabetes as well as arthritis. Fabaceae family was dominating. Antioxidant, anti-inflammatory, cytotoxicity, alpha-amylase and alpha-glucosidase) of five plant species, (*Bridellia mollis, Elephantorrihiza burkei, Elaeodendron transvaalense, Senna petersiana* and *Searsia lancea)* used traditionally to manage diabetes were investigated using the standard *in vitro* procedures.

All extracts showed a good nitric oxide inhibition, with highest percentage inhibition found in the highest concentration of 100 µg/ml. They all had good percentage cell viability at lowest concentration which was comparable to quercetin. Only two plant extracts *B. mollis* (T2) and *E. transvaalense* (T3) had lower than inhibition of quercetin at 25 µg/ml than at 12.5 µg/ml. In vero cells low toxicity effect was observed at lowest concentration tested, and toxicity increased with the increase in concentration. In bovine dermis cell line all plant extracts had more cell viability at lower concentration than doxorubicin. Ethanol extracts of *B. mollis* and *S. petersiana*, and ethyl extract of *E. transvaalense* had a good alpha-amylase inhibitory activity with IC_{50} values 58.6, 81.9 and 131.5 mg/ml respectively. Hydro-ethanol, ethyl acetate and ethanol extracts of *E. burkei* exhibited a significant alpha-glucosidase inhibitory activity with IC_{50} values 56.9, 52.2 and 129.7 mg/ml respectively. Kinetic analysis revealed non-competitive and un-competitive inhibitions of the plant extracts on alpha-amylase and alpha-glucosidase enzymes respectively.

The information obtained showed that people in Vhembe District Municipality still rely on medicinal plants to treat and manage diabetes and arthritis. All plant extracts were toxic to both bovine dermis and vero cell lines. *S. lancea* (T5) was found to be the most toxic plant extract. The observed good inhibitions of both alpha-amylase and alpha-glucosidase enzymes by plant extracts of *B. mollis, S. petersiana, E. transvaalense* and *E. burkei* validate their use in the traditional treatment of diabetes in the region to some extent. Aqueous extracts of these medicinal plants should also be investigated because water is the main solvent which is used by traditional practitioners in the preparation of their herbal medicines.

Keywords: Medicinal plants; diabetes; arthritis; anti-inflammatory; cytotoxicity.

Conference contribution

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Declaration

I, **Pfarelo Witney Tshidzumba**, declare that this dissertation is my original work and has not been submitted for any degree at this or any other university or institution. The dissertation does not contain other person's writing unless specifically acknowledged and referenced accordingly.

Signed (Student): ..... Date: .....

DEDICATION

I dedicate my dissertation work to my family, friends, my brothers and everyone who played a role in my upbringing. A special feeling of gratitude to my mother, Miss Tshidzumba Livhuwani, for her Words of encouragement, her unconditional love, always pick me up whenever I`m down, and many sacrifices she made for me in my lifetime to help me fulfill my dream and become who I am today, big thanks goes to my life partner and friend Mathidi Mpho Ralph for his words of encouragement, his everlasting love and support. And lastly to my daughter Oritonda Mathidi you have been the source of my motivation during last and difficult moments.

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List of abbreviations used

Abs-	Absorbance
ABTS-	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
CV-	Cardiovascular
BD-	Bovine dermis
DM-	Diabetes mellitus
DMEM-	Dulbecco`s modified eagle medium
DMSO-	Dimethylsulfoxide
DNS-	Dinitro salicylic acid
DOXO-	Doxorubicin
PNPG-	p-Nitrophenyl glucopyranoside
DPPH-	2, 2- diphenyl-1-picrylhydrazyl
ET-	Electron transfer
FCS-	Foetal calf serum
HAT-	Hydrogen atom transfer
IC50-	Inhibition concentration
LPS-	Lipopolysaccharide
MD-	Maryland
MEM-	Minimum essential medium
MTT-	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NO-	Nitric oxide
NOS-	Nitric oxide synthases
NTLC50-	Non-toxic limit concentration
COPD-	Chronic obstructive pulmonary disease
pH-	potential of Hydrogen
PSF-	Penicillin/streptomycin/fungizone
RSA-	Republic of South Africa
USA-	United States of America
WHO-	World Health Organization

Chapter 1: Introduction

1.1 Introduction

For a long period of time, plants have been a valuable source of natural products for sustaining human health (Chandrakar *et al*., 2013). Their products have been used in the traditional medicinal system for treatment of various diseases; today plants are being extensively explored in search for medicinal properties (Kumar *et al*., 2010). Plants have contributed extremely to Western medicine, through providing ingredients for drugs and they have played huge role in drug discovery (Atanasov *et al*., 2015).

Estimates suggest that up to 80% of the population in Africa uses traditional medicine as their primary source of health care (WHO, 2002). Approximately 3000 plant species are utilized for medicinal purposes by an estimated 200 000 indigenous traditional South African healers (van Wyk *et al*., 2009). South African's rich plant diversity is a source of herbal medicines and their uses in the treatment of various ailments has been part of human civilization since ancient times (Van der Merwe *et al*., 2001).

In Venda, the use of medicinal plants is a common practice. It is influenced by local traditions as well as the availability of highly diverse medicinal resources (Samie *et al*., 2010). Herbal medications have been employed to care for numerous persistent and incurable diseases in other organizations of medicine. People are getting more familiar with them because of their several perceived advantages like fewer side effects, better patient tolerance and being relatively cheaper (Masevhe *et al.,* 2015).

The focus of this study was to identify and record plants which are used to treat and control diabetes and arthritis in Vhembe District Municipality as well as evaluating their anti-inflammatory, anti-diabetic and cytotoxicity activity. In this dissertation chapter 2, 3, and 4 have been written in manuscript format, chapter 1 and 5 have been written in a traditional style, as such there will be a repetition of some concepts in different chapters.

1.2 Aim

The study was intended to document medicinal plants used to treat and manage diabetes and arthritis in Vhembe District Municipality as well as evaluating their *in vitro* efficacy.

1.3 Objectives

The following objectives were considered in order to accomplish the aim;

- To record and identify medicinal plants used to treat and control diabetes and arthritis.
- To determine the antioxidant activity of the plant extracts.
- To evaluate the *in vitro* anti-inflammatory activity of the plant extracts.
- To evaluate the *in vitro* alpha-amylase and alpha-glycosidase inhibitory activities of the plant extracts.
- To determine the cytotoxicity of the plant extracts.

1.4 Study hypotheses

- Vhembe District local people depend on the use of medicinal plants as their primary source of health care in the treatment of diabetes and arthritis.
- The constituents of plants used in traditional medicine possess therapeutic effects such as anti-inflammatory and anti-diabetic activities that can help in alleviating diabetes and arthritis in humans.

1.5 Rationale of the Study

The indigenous knowledge which has been orally transferred from generation to generation is fast disappearing because of the technology and cultural changes in ethnic groups. It is for the same reason ethnomedicinal study and its restoration is very vital. This kind of study has a potential for discovery of more successful medicines. There are almost half a million plants around the globe, and most of them have not been investigated in terms of medicinal activities (Rasool, 2012). Therefore, it is important to gather, document and develop indigenous information from knowledge holders. Accessibility of modern medicine is also a problem in Vhembe Local Municipality as most people reside in the rural area where they have to walk for long distances to get general health care. As a result local people are highly dependent on medicinal plants to treat diabetes, arthritis as well as other ailments.

1.5 Literature Review

1.5.1 Chronic illnesses

Chronic diseases are diseases that lasting for a long time, which cannot be cured or vaccinated. They are significant to the public health worldwide with an estimation of trillion dollars in annual health care cost and claiming the spirits of more than 36 million people in a single year, the growth in the rate of these diseases has created a massive social, economic, and emotional, burden that is prevailing all over the globe (Durstine *et al.,* 2013). Chronic diseases are increasing due to the rapid aging of the population and the greater longevity of people with chronic conditions (van Oostro *et al.,* 2016). Common chronic conditions include arthritis, asthma, cancer, chronic obstructive pulmonary disease (COPD), diabetes and viral diseases such as HIV/AIDS and hepatitis C (Bernell and Howard*.,* 2016). However, the focus of this work is on two chronic conditions, namely arthritis and diabetes.

1.5.2 Arthritis

Arthritis is an inflammatory disease characterized by the damage to the articular cartilage, changes in subchondral and marginal bone, synovitis and capsular thickening and it usually affects the weight bearing joints (Wambugua, 2011). It is a leading cause of pain and disability worldwide (Benjamin *et al.,* 2014), as well as a major public health problem due to its high prevalence and costs (Srikanth, 2005). Arthritis affects every population and ethnic group, but it commonly affects elderly populations (Gabriel and Michaud*,* 2009).

The World Health Organization (WHO) estimates that globally, approximately 25% of adults aged over the age of 65 years suffer from pain and disability associated with arthritis (Breedveld, 2004). The etiology and mechanisms responsible for the development of arthritis are still not very clear (Srikanth, 2005). The main risk factors associated with arthritis are age, gender (more frequent in women), obesity, metabolic endocrine diseases, trauma or joint overload, and genetic factors (Cunha-Miranda *et al.,* 2015). There are six types of arthritis, namely; Rheumatoid arthritis, osteoarthritis, juvenile arthritis, psoriatic arthritis, gouty arthritis as well as ankylosing (Symmons *et al.,* 2006).

Development of arthritis cannot be prevented, but symptoms can be relieved and joint functioning can be improved (Woolf *et al.,* 2003). There are four main control categories of arthritis: non-pharmacologic, (weight loss and exercise), pharmacologic which include complementary, alternative, and surgical, but treatment should begin with the safest and least invasive therapies before more invasive and expensive ones (Keith *et al.,* 2012).

So far there is no specific drug which is specifically effective in the treatment of arthritis (Xie *et al.,* 2015). Acetaminophen/paracetamol is used as first line and NSAIDs (non-steroidal anti-inflammatory drugs) are only recommended as add-on treatment if pain relief is not sufficient, due to their relative greater safety (Wambugua, 2011).

All these treatments are only capable of easing pain as well as cutting down the progression, and improving the function of affected joints, but they do not reverse the damage to joints (Coman *et al.,* 2012). Nonsteroidal anti-inflammatory drugs and selective Cyclo-Oxygenase-2 inhibitors, which are orally taken or applied are recommended for patients who are not responding to acetaminophen. Unfortunately, these medications can cause severe and adverse side effects like gastrointestinal haemorrhage (Fox and Stephens, 2010), cerebrovascular diseases (Xie *et al.,* 2015), liver dysfunction and other side effects (Nagori *et al.,* 2010), which are life threatening (Wambugua, 2011).

Medicinal plants contain compounds that have the potential to heal, maintain health as well as prevent diseases (Rios and Recio*,* 2005). Therefore uses medicinal plants has gained popularity globally in the last decade (Masoko *et al.,* 2013). This might be because of their low toxicity and less side effects (Gupta *et al.,* 2012). Medicinal plants are dependable, comparatively cheap, and convenient for many patients (Nagori *et al.,* 2010). These plants are used on their own as a primary therapeutic choice, or mixed with other conventional medications (Ezuruike and Prieto, 2014). Natural compounds have been used since time immemorial for treatment of diabetes related symptoms, have been proven to reduce the risk of disease, and can be daily ingested at larger quantity (Coman *et al.,* 2012).

People use medicinal plants and other therapies to treat diabetes since most of the conventional drugs are either costly or not accessible (Shinkafi *et al.,* 2015). Medicinal plants such as *Psidium guava* leaves and fruits have been established to have anti-diabetic properties (Marikandan *et al*., 2013), as well as *Prunus persica* leaves (Wadood *et al*., 2013), *Capparis tomentosa* roots (Wangai *et al.,* 2015) and *Momordica foetida* leaves (Acquaviva *et al.,* 2013). Plants such as *Sida cordifolia* barks have been used to treat arthritis and other illnesses such as heart and nutritional diseases (Lakhande *et al*., 2006). Plants such as *Holarrhena pubescence* seeds are found to have antioxidant actions in their stem barks (Bhusal *et al*., 2014), *Prunus persica* stem bark (Ruturi *et al.,* 2011), *Psidium guajava* leaves (Balakrishna *et al.,* 2011), and *Salix mucronata* bark (Van Wyk *et al.,* 2007).

1.5.3 Diabetes mellitus

Diabetes mellitus (DM) is a chronic endocrine disorder that involves the metabolism of sugars, proteins, fat and electrolytes. It is characterised by elevated blood sugar level, which is triggered by low insulin production or not responding to insulin produced (Nair *et al.,* 2013). It is one of the most prevalent chronic diseases all over the world contributing significantly to the worldwide burden of diseases (Falconer *et al.,* 2014). Diabetes prevalence is increasing in developed and developing countries (Gomes *et al.,* 2012). The global prevalence of diabetes is estimated at 6.4% and 3.8% in the African region (Rengasamy *et al.,* 2013). In South Africa, adults between the ages of 20-79 years are diabetic, and this is projected to increase from 1.9 million in 2011 to 2.5 million in 2030 with approximately 78% being undiagnosed (Mabaso *et al.,* 2014).

There are several pathogenic processes that are involved in the development of diabetes. These include autoimmune destruction of the β-cells of the pancreas with a consequent insulin deficiency to abnormalities that result in resistance to insulin action (Surya *et al.,* 2014). DM is caused by both genetic and environmental factors (Topf *et al.,* 2013). There are two types of diabetes. Type-1 diabetes is an autoimmune disease characterized by T-cell mediated destruction of the pancreatic beta cells. Failure of the beta-cell secretory machinery has been suggested as a primary cause for the reduced insulin secretion (Topf *et al.,* 2013). This type of diabetes commonly develops in childhood and progresses with age. Type 2 DM is the most common type of diabetes, which is previously known as non-insulin dependent DM or maturity-onset diabetes. There are two metabolic defects which characterize this type of diabetes; insulin resistance and inadequate insulin secretion (Surya *et al*., 2014). This type of diabetes affects around 90%-95% of all patients (Mabaso *et al.,* 2014). The most common symptoms of DM include an excessive urge to eat, passing large amounts of urine within short intervals, severe thirst, poor vision and weight loss, but some people might not show any symptom (Semenya *et al*., 2012).

Current therapies which are available for diabetes are insulin and various oral anti-diabetic agents such as sulfonylureas, biaguanides, glinides, insulin analogues, glucagon-like peptide 1 (GLP-1). Agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors (Ezuruike and Prieto, 2014), only have short term effects of inhibiting enzymes to decrease high glucose levels in the blood (Nair *et al*., 2013). All these drugs have a number of undesired gastrointestinal side effects such as diarrhoea, gastritis, abdominal bloating, limited efficacy and limited tolerability based on their working mechanisms (Patel *et al.,* 2012).

Chapter 2: An inventory of medicinal plants used as anti-diabetes and anti-arthritis in the Vhembe District Municipality, Limpopo Province, RSA

Abstract:

The aim of the study was to document medicinal plants which are used to treat and manage diabetes as well as arthritis by traditional healers, herbalists and elderly people in the Vhembe District Municipality. Traditional healers, herbalists and elderly people were interviewed using semi-structured questionnaires. Seventeen plant species belonging to fourteen different families were found to be used in the treatment of diabetes as well as arthritis. Fabaceae was the most dominating families with three plant species followed by Cucurbitaceae with two plant species and the remaining families had one plant species each. Most people use tree species more than other plant forms such as shrubs, climbers and herbs. The leaves were highly mentioned to be used for the treatment of diabetes. The most frequently used preparation mode of medicine has been decoction (17), followed by soaking (3) and burning (2). The most preferred administration mode was oral (14), followed by bathing (5), topical (2) and the least used mode was heat fumigation (1). The information obtained showed that traditional health practitioners of Vhembe District Municipality still rely on medicinal plants to treat and manage diabetes and arthritis.

Keywords: Medicinal plants; traditional healers; diabetes; arthritis; Vhembe District Municipality.

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2.1 Introduction

For a long period of time, plants have been a valuable source of natural products for maintaining human health (Dwivedi *et al.,* 2014). Estimates indicate that ± 80% of the population in Africa makes use of traditional medicine as their primary source of health care (Manjula and Mamidala, 2013), and about 80% of South Africa population consult traditional healers (Magwede *et al.,* 2014). South Africa is a home to more than 24 000 plant species almost 10% of all plant species in the world (Low and Rebelo, 1996). This flora diversity is a rich source of herbal medicines for indigenous South African people and the use of these medicinal plants in the treatment of various ailments has been part of human culture since ancient times (Van der Merwe *et al.,* 2001). In Venda, the use of medicinal plants is very common, based on local traditions as well as the availability of highly diverse genetic resources (Samie *et al*., 2010). It is estimated that up to 700,000 tonnes of plant material are consumed annually and make a value of about 150 million US dollars (Street and Prinsloo, 2012). Plants possess anti-microbial, anti-viral, anti-cancer, anti-inflammatory, anti-diabetic, hemolytic, antioxidant and larvicidal properties (Kumar *et al.,* 2010), all these phytoconstituents are important for the good function the body.

Documentation of indigenous and traditional knowledge is very crucial for future studies which will lead to sustainable utilization of natural resources (Singh *et al.,* 2012), thereby helping people realize the importance of maintaining biodiversity. There is a need of detailed documentation on the use of medicinal plants in South Africa (Masevhe *et al*., 2015). If we are to study the use of medicinal plant we can surely overcome the need of modern doctors in the world were the proportion of medical doctors to patients in Africa is South Africa 1:1639; Ethiopia 1:33,000; Kenya 1:7142; Tanzania 1:33,000; Uganda 1:25,000, Malawi 1:50,000; Mozambique 1:50,000 and Swaziland 1:10,000 (Masevhe *et al.,* 2015). The focus of this work was to identify and to record plants used to treat and control diabetes as well as arthritis in Vhembe district municipality. This is the first study to document the use of medicinal plants in the treatment of both diabetes and arthritis in Vhembe District Municipality. However, there is some work which has been done to document the use of plant in the treatment of diabetes in South Africa by Deutschländer *et al.* (2009), Oyedemi *et al.* (2009) and Semenya *et al.* (2012). There is also some work done by Dzoyem and Eloff. (2014), Adebayo *et al.* (2015), Elisha *et al,* (2016), on plants used as anti-inflammatory in South Africa.

2.2 Materials and methods

2.2.1 Study area

Vhembe District Municipality is 25 597km², located in the northern part of the Limpopo Province, It is one of Limpopo province's six district municipalities, namely Mopani, Capricorn, Waterberg, Bohlabela and Sekhukhune (www.vhembe.gov.za). The district has four local municipalities, namely: Musina, Mutale, Thulamela and Makhado municipality. It is comprised of three ethnic groups: Vhavenda, Pedi and Tsonga. The majority of 1.2 million of people are Venda speaking (Luseba and Tshisikhawe, 2013).

It covers a geographical region that is predominantly rural. The vegetation of the area includes two vegetation types within the savannah biome; mixed Lowveld Bushveld and Mopani Bushveld (Magwede *et al.,* 2014). The area has a wet and hot summer with a mean temperature of 30˚C, and a dry and cool winter with a mean temperature of 18˚C, humidity in the area is ±40% (Luseba and Van der Merwe, 2006).

2.2.2 Maps of the study area

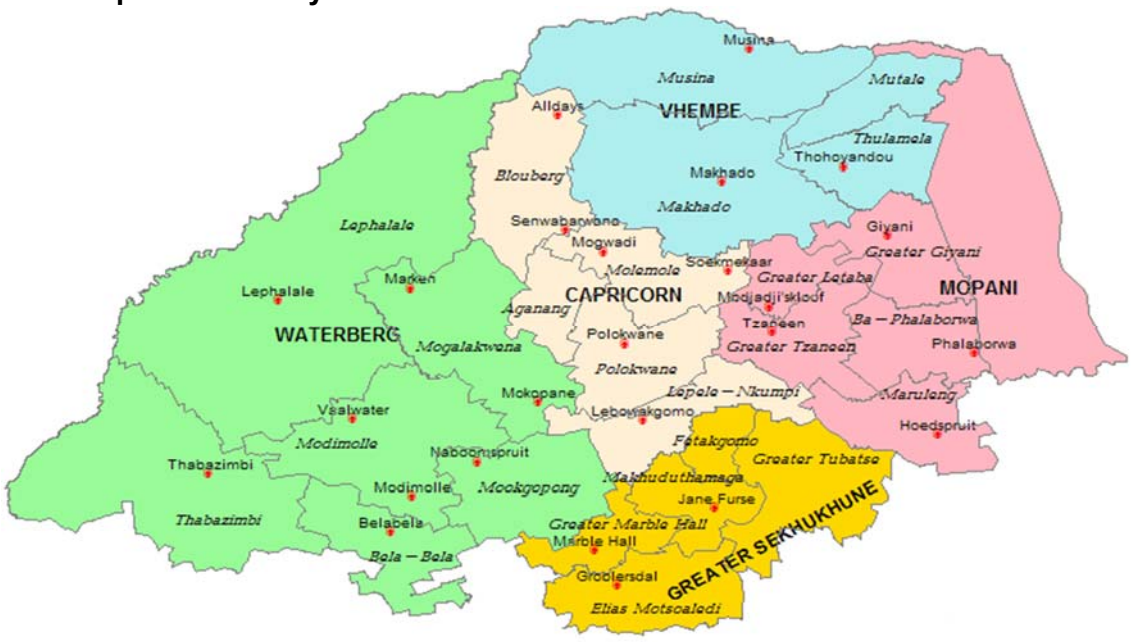


Figure 1. Map showing where Vhembe District Municipality is located in Limpopo province (www.slideplayer.com)

Figure 2. Map of Vhembe District Municipality and its four local municipalities. (www.vhembe.gov.za)

2.2.3 An ethnobotanical approach used to select plants for the study

An ethnobotanical study was conducted in the Vhembe local municipality in three different local municipalities; Makhado, Thulamela and Mutale. The study area was visited several times in September 2014 to May 2015. The ethnobotanical survey was conducted after seeking permission from the local authorities; participants were randomly selected, but participation in this study was voluntary. Only a few people were willing to participate even after great efforts were made to convince them about the importance of documenting such information. The main purpose of an ethnobotanical survey was explained to the traditional healers before the interview was conducted.

The information on medicinal plants that are used for the treatment of diabetes and arthritis in the area was collected from traditional practitioners, herbalists and elderly people, using a questionnaire (Appendix 1). Interviews were conducted in their homesteads using the local language. Information obtained was recorded with Phillips voice tracer (DVT 1150) and later translated into English.

The recorded information was expressed as a “frequency index” (FI), together with additional information such as vernacular names of the plants, parts used (Table 1 and 2). The frequency of use was calculated using the formula: Frequency of use (FI=FC/N*100) N=10 which is the total number of informants, FI=is the percentage and FC=is the number of informants who mentioned it (Mahwasane *et al.,* 2013). Frequency index is high when there are many informants who have mentioned a particular plant and low when there are few reports. Plant species were identified and voucher specimens were deposited at University of Venda herbarium.

2.3 Results and discussion

2.3.1 Plants reported to be used to treat diabetes and arthritis in Vhembe District Municipality.

Vhembe District Municipality is situated at the northern part of the Limpopo Province, It shares borders with Zimbabwe and Botswana in the north-west and Mozambique in the south-east, there are few health care facilities scattered around the area, but some people still use medicinal plant as their primary source of health care.

Information on plants recorded that are used for treating arthritis and diabetes in Vhembe District Municipality recorded during the survey is listed in (Table 1 and 2) respectively. For each species the following ethnobotanical information was provided: family, botanical and vernacular names, voucher specimen numbers, plant parts used, plant form, availability of plant species, modes of preparation, as well as the frequency index, and IUCN Conservation status. For those plants which were only provided in the vernacular name book and oral consultations were used to obtain the scientific names.

2.3.2 Ethnobotanical information of plants used to treat diabetes and arthritis

A total of 10 informants were interviewed about the medicinal plants which are used to treat and control diabetes. Seven of the informants were females with their ages ranging from 57 years to 98 years, 3 were males with their ages ranging from 35 years to 42 years. These results showed that women know more about the uses of medicinal plants than men. A study done by Shosan *et al*. (2014), also shows that out of fifty people interviewed, 20% were males and female were 80% of knowledge holders. Tahraoui *et al*. (2007), report that women are the majority consumers as well as the prescribers of medicinal plants. The fact that women are usually out in the fields collecting firewood, cow dung and food like vegetables, mushrooms (Fartyal *et al.,* 2013), might be the contributing factor as the medicinal plants are commonly found in the fields.

Some plants such as *Bridelia mollis*, *Aloe micracantha* and *Elephantrorriza burkei* were recorded for the first time in treatment of diabetes. *Commiphora viminea, Elephantrorriza burkei* and *Senna petersiana* were also recorded for the first time in the treatment of arthritis. Some of the plants were prepared combined with others, for example, plants such as *Psidium guajava* and *Elaeodendron transvaalense* were mixed to treat diabetes, *E. burkei*

and *S. lancea* were prepared as mixture for the treatment of diabetes and arthritis or each plant was prepared on its own to treat both diabetes and arthritis. This is a common practice in traditional medicine preparation, it is either to make the mixtures more potent, and to mask or reduce toxicity (Madikizela *et al*., 2012).

Conservation of indigenous plant species around the world is one matter which has been going on for decades, yet plants continue to decline at an alarmingly higher rate (van Breugel *et al.,* 2011). It was upsetting to hear informants mentioning that they were not doing anything to conserve these plants, Nowadays medicinal plants are being gathered by people with no plant conservation knowledge, unlike previously, where harvesting of medicinal plants was done by well-developed traditional medical practitioners, with particular skills (Williams *et al*., 2000). After using IUCN (2011) red data list, it is worth to mention that 12 medicinal plants are least threatened, 2 are not threatened, namely *A. micracantha* and *E. traansvaalense*. *P. guajava* and *P. Persica are regarded as plants of least concern by IUCN (2011),* this was worrisome because they have scored high frequency of used (Table2). It implies that they are highly used, and if they are highly used and follow under plants of concern they might be endangered in the near future or face extinction caused by overuse. Only *P. guajava* high frequency index might be beneficial since this plant is regarded as highly invasive and pollutant (Semenya et al., 2012).

Table 1. Frequency index of ethnobotanical plants used for the treatment of arthritis

Plant names &families	**vernacular name**	**Voucher no.**	**Plant Form**	**Part used**	**Frequency**	**Preparation**	**Availability**	**IUCN status**	**Reported Anti-inflammatory usage**	**Reported Anti-diabetics usage**
Bridelia mollis Hutch Euphorbiaceae	mukumba kumba	T3	S	L	10	Boiling	H	LC	No report	No report
Capparis tomentosa Lam Cappraceae	muombadali	T7	T	R	20	Boiling	L	LC	Leaves used for inflammation (Hurinanthan, 2009)	Roots used for diabetes (Wangai *et al*., 2015)
Colophospermum mopane (J. Kirk ex Benth.) J. Léonard Fabaceae	mupani	T8	T	R	20	Soaking	H	LC	No report	No report
Commiphora viminea Burtt Davy Burseraceae	mutahadzi	T10	T	B	20	Soaking	L	LC	No report	No report
Elaeodendron traansvaalense Burtt Davy) R.H.Archer Celestraceae	mukuvha zwivhi	T3	T	SB	30	Boiling	VL	NT	No report	Bark extracts used for diabetes (Deutschländer *et al.,* 2009).
Elephantrorriza burkei Benth Fabaceae	gumululo	T2	S	B	20	Soaking	H	LC	No report	No report

Ficus ingens (Miq.) Miq Moraceae	mukululu	T11	T	SB	10	Boiling	VL	LC	Roots used for inflammation (Maroyi, A. 2013).	No report
Moringa oleifera Lam Moringaceae	muringa	T12	T	L	10	Soaking	M	NA	Seeds used for inflammation (Minaiyan *et al.,* 2014); Leaves (singh *et al.,* 2012)	Leaves used for diabetes (Luangpiom, 2013); Seeds (Al-Malki and El Rabey, 2014)
Searsia lancea (L.f.) F.A.Barkley Anacardiaceace	mushakaladza	T5	T	R	10	Soaking	H	LC	No report	No report
Scleorrocarrya birrea (A.Rich.) Hochst. Anacardiaceace	mufula	T16	T	B	10	Boiling	L	LC	Roots used for inflammation (Maroyi, A., 2013).	Roots used for diabetes (Maroyi, A., 2013).
Senna petersiana (Bolle) Lock Fabaceae	munembenembe	T17	S	F& R	10	Soaking	H	LC	No report	No report

Key: T-tree, S-shrub, H-herb, C-climber, L-leaves, B-bark, R-roots, L&B-leaves and barks, B-bulb, L&SB- leaves and stem bark, F-fruit, SB-stem bark, VL-Very low, L-low, M-medium, H-high, LC-least concern, and NT-not threatened, NA-not yet assessed, DD-data deficiency

Table 2. Frequency index of ethnobotanical plants used for the treatment of diabetes

Plant names &families	vernacular name	Voucher no.	Plant Form	Part used	Frequency	Preparation	Availability	IUCN status	Reported Anti-inflammatory usage	Reported Anti-diabetics usage
Aloe micracantha Haw Liliaceae	tshikhopha tshituku	T6	H	L	10	Soaking	L	NT	No report	No report
Bridelia mollis Hutch Euphorbiaceae	mukumba kumba	T3	S	L	10	Boiling	H	LC	No report	No report
Combretum molle R.Br ex G. Don Combretaceae	mugwiti	T9	T	L&SB	10	Soaking	M	LC	Leaves used for inflammation (Bessong *et al*., 2006); Roots (Eloff *et al*., 2005).	Leaves used for diabetes (John *et al*., 2009)
Cucurbita pepo (Linnaeus) Dumortier Cucurbitaceae	thanga	T11	C	F	10	Boiling	H	LC	Seeds used for inflammantion (Malgwi *et al*., 2014); Leaves (Oloyede, 2012)	Fruits used for diabetes (Kwiri *et al*., 2014); Leaves (Froelich *et al.,* 2007); Seeds (Malgwi *et al*., 2014); Leaves (Oloyede, 2012).

Elaeodendron traansvaalense Burtt Davy) R.H.Archer Celestraceae	mukuvha zwivhi	T3	T	SB	30	Boiling	VL	NT	No report	Bark extracts used for diabetes (Deutschländer *et al.,* 2009).
Elephantrorriza burkei Benth Fabaceae	gumululo	T2	S	B	20	Soaking	H	LC	No report	No report
Mormodica foetida Schumach Cucurbitaceae	nngu	T13	C	L	10	Boiling	H	LC	No report	Leaves used for diabetes (Acquaviva *et al*., 2013).
Prunus persica (Linnaeus) Batsch Rosaceae	muberegisi	T14	T	L	30	Boiling	H	LC	Stem bark is used for inflammation (Raturi *et al*., 2011)	Leaves used for diabetes (Wadood *et al*., 2013)
Psidium guajava Linn Myrtaceae	mugwavha	T15	S	L	30	Boiling	H	LC	Leaves used for inflammation (Balakrishnan *et al*., 2011).	Fruits used for diabetes (Rai *et al.,* 2009; *Rapaka et al., 2012)*; Leaves *(*Manikandan *et al.,* 2013)
Searsia lancea (L.f.) F.A.Barkley Anacardiaceace	mushakaladza	T5	T	R	10	Soaking	H	LC	No report	No report
Senna petersiana (Bolle) Lock Fabaceae	munembenembe	T17	S	F& R	10	Soaking	H	LC	No report	No report

Key: T-tree, S-shrub, H-herb, C-climber, L-leaves, B-bark, R-roots, L&B-leaves and barks, B-bulb, L&SB- leaves and stem bark, F-fruit, SB-stem bark, VL-Very low, L-low, M-medium, H-high, LC-least concern, and NT-not threatened, NA-not yet assessed, DD-data deficiency

Three families were found dominating Fabaceae at 17%, followed by Cucurbitaceae and Anacardiaceace both at 11%. Arabi and Sardari. (2010), have found the Fabaceae family to be dominating. This is not surprising since Fabaceae is the third largest family of angiosperm plants with approximately 730 genera and over 19,400 species worldwide (Masevhe *et al.,* 2015).

Trees were highly used (56%) as compared to shrubs (28%), climbers (11%) and herbs (5%). Mustapha (2014) also found that trees are the preferred plant form; this might be because trees are available throughout the year. Trees are resistant to drought and seasonal changes (Maroyi, 2013). It is reported by Masevhe *et al.* (2015), that trees usually bear greater quantities of compounds such as phenols, tannins, alkaloids, triterpenes and quinones than shrubs and herbaceous species. The leaves were found to be most frequently used, followed by roots/bulbs and bark in the treatment of both arthritis and diabetes, fruits and seeds were least preferred.

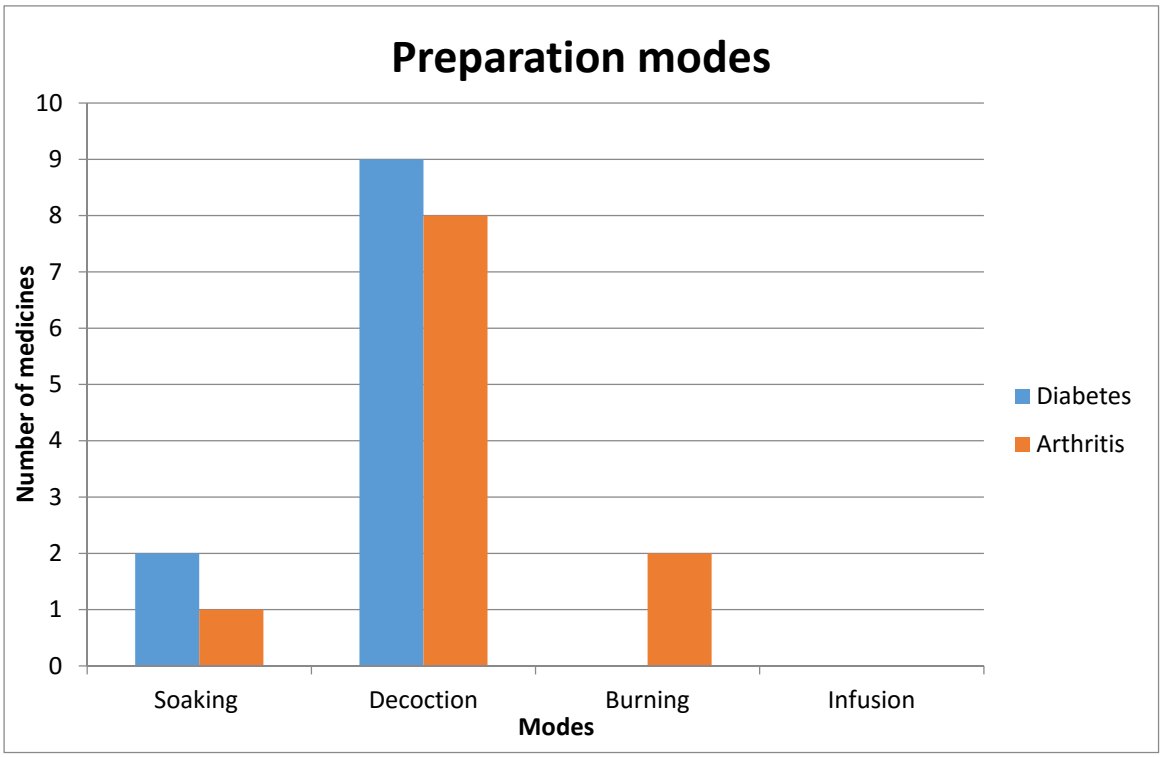


Figure 3. Preparation modes

Figure 3 shows preparation modes of medicinal plants, the most medicines were prepared by decoction 17, followed by soaking 3. The fact that most medicine were prepared by decoction indicated that the local healers have an indigenous knowledge that partially shares the modern knowledge of drug preparation methods of effective treatment (Wabe *et al*., 2011). Most modern medicines are prepared by soaking and decoction (Shosan *et al.,* 2014). Some plant materials were ground into fine powder then mixed with soft porridge and given to a patient to eat. Very few plants were burnt (2) and rubbed on the patient's body and that was only found in plants which were used in the treatment of arthritis. Traore *et al,* (2013) has reported that most remedies are prepared by decoctions and soaking. These two methods were found to be used in both arthritis and diabetes.

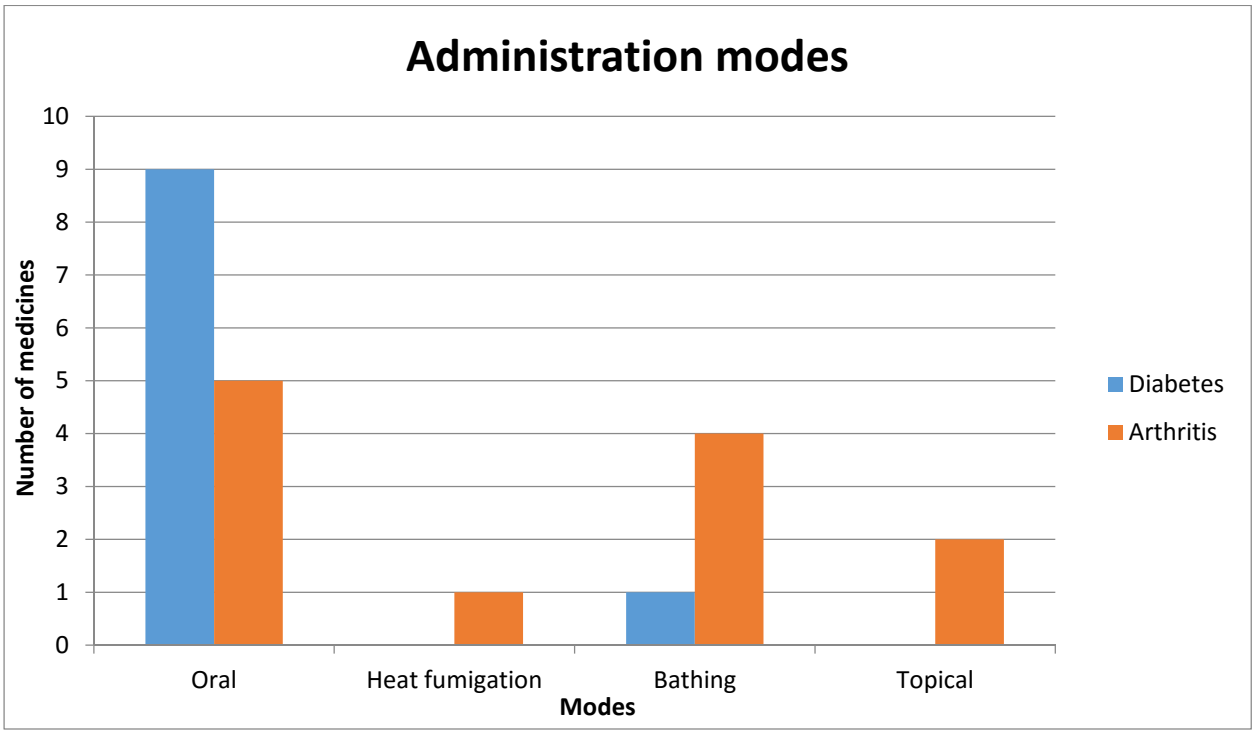


Figure 4. Administration modes

Figure 4 indicates administration modes of medicinal plants by Vhembe district municipality local people. Oral (14) administration was the most preferred method in both diabetes and arthritis. Decoction was drunk or medicinal plant was mixed with soft porridge and eaten. The second most preferred method was bathing (5) followed by topical (2). Plant materials were burnt to ashes, then mixed with animal fats or petroleum jelly and used to massage the patient. The least used method was heat fumigation (1). Out of the four preferred methods, three were highly used in the treatment of arthritis, topical (2), heat fumigation (1) and bathing (4) this might be because arthritis affects bones. Oral (9) administration was commonly used in the treatment of diabetes, topical and heat fumigation was not used in diabetes.

The least commonly used method was heat fumigation (1), which was only used in arthritis in one medicine. In this mode plants were boiled in water, then the patients were covered by blanket with the hot mixture inside, the patient will inhale the mixture with steam and sweat. Oral administration was most popular as it was used in the treatment of both diabetes and arthritis; this might have been influenced by the fact that most medicines are administered this way as they will easily absorbed by the body faster. Ndipa *et al.* (2013), mention that DM is metabolic disorders in which the blood sugar is higher than normal level. Consumed medication might be easily be soaked up from the stomach to the blood fats in high dosage.

2.3.3 Botanical description, other medicinal uses, active components and pharmacological effects of highly mentioned plants for treatment of diabetes and arthritis in Vhembe District Municipality, Limpopo province.

This section discusses three selected plants that were stated in the treatment of diabetes and arthritis in Vhembe District Municipality. *Prunus persica, Psidium guajava* and *Eleodendron transvaalense* were selected because they had higher frequency index.

Prunus persica (Linn)

Botanical description

Prunus persica is a deciduous tree which grows up to 10 m high (Raturi *et al.,* 2011). The tree originated from Central Asia, is now growing in many subtropical and temperate areas of the world (Ning *et al*., 2004). Commercially cultivated peach tree has bark, which is grey or ashy or serrate acuminate glabrous. Flowers are pinkish white in colour, and short.

This tree is commonly cultivated for its juicy fruits (Raturi *et al.,* 2011). According to International Union for Conservation of Nature (2011) data list. (IUCN) *P. persica* species is least threatened.

Other medicinal uses

The leaves are used as astringent, demulcent, diuretic, expectorant, febrifuge, laxative, parasiticide and mild sedative. They are used internally in the treatment of gastritis, whooping cough, coughs and bronchitis (Raturi *et al.,* 2011). Leaf paste is applied to treat fungal infection as well as treating worms on wounds. Leaves are orally taken for treatment of gastritis, whooping cough and chronic bronchitis. Flowers are considered as laxative and diuretic and are used to treat constipation and oedema (Aziz and Rahman*,* 2013).

Active components and pharmacological effects

Prunus persica is well known for several activities such as antibacterial, antifungal, antioxidant and antiviral (Raturi *et al.,* 2011), anti-fungal activities (Wadood *et al.,* 2013). Leaves of *P. persica* showed significant antihelmintic activities in the study done by Usharani *et al.* (2014). Seeds had anti-inflammatory and antitumor activity, leaf extracts showed antihyperglycemic activity, (Han *et al.,* 2015). It contains components such as sugars, proteins, fats, glycosides, flavonoids, alkaloids, essential oils, tannins, mucilages, pectins, minerals and vitamins (Malinowska, 2016). The fruit is used as a demulcent, an anti-scorbutic and a stomachic. It fruits contain two Flavan-3-ols, flavonols and quercetins (Zhao *et al.,* 2015). This species is also known to have aphrodisiac, anti-pyretic properties. The seeds have an anthelmintic and emmenagogue properties (Aziz and Rahman, 2013). *P. persica* essential oil has been found to be non-toxic to mammalian cells, but they had a unique fungitoxicity (Tripathi, 2016).

Psidium guajava (Linn)

Botanical description

Psidium guajava is a shrub which spread to various parts of the tropical and subtropical regions (Santhoshkumar *et al*., 2014). It bears globous or piriform fruits, which are yellow in colour 3-6 cm in diameter (Braga *et al.,* 2014). It is distributed from México down to São Paulo State, Brazil; it is indigenous to Central América and part of South America between

Colombia and Peru (Gonçalves *et al.,* 2008). It is well known for its edible fruit. It is a common backyard tree with crooked branches and opposite leaves. The flowers are white, incurved petals, 2 or 3 in the leaf axils; they are fragrant, with four to six petals and yellow anthers (Orwa *et al.,* 2009). According to International Union for Conservation of Nature (2011) data list (IUCN) *P. guajava* is least threatened.

Other medicinal uses

Leaves of *P. guajava* are used as remedy for diarrhoea (Rapaka *et al.,* 2012). In Trinidad, a tea made from young leaves is used for diarrhoea, dysentery and fever; extracts of roots, bark, and leaves are used to treat gastroenteritis, vomiting, diarrhoea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, and a number of other conditions (Chetia *et al.,* 2014). The leaves are commonly used as popular medicine for rheumatic pain; they are chewed to relieve toothache (Santhoshkumar *et al.,* 2014). Different parts of the plant are used in folk medicine in the treatment of diseases such as lesions, cholera, hypertension, obesity and the control of diabetes mellitus.

Active components and pharmacological effects

Psidium guajava has been reported to have anti-diarrheal, anti-inflammatory and anti-cancer activities (Santhoshkumar *et al.,* 2014). The main components are quercetin (Santhoshkumar *et al.,* 2014), vitamins, tannins, phenolic compounds, flavonoids, essential oils, sesquiterpene alcohols and triterpenoid acids (Barbalho *et al*., 2012). The leaves have been reported to have anti-bacterial properties (Gonçalves *et al.,* 2008) antitumor, antioxidants and anti-cariogenic (Braga *et al*., 2014). Essential oils from guava leaves displayed anti-cancer activity *in vitro* (Chetia *et al.,* 2014). In India, the young leaves are used as antitussive. In China, the leaves are used as anti-inflammatory and hemostatic agent (Braga *et al.,* 2014). It has toxicity against human colon cancer cells (Lee and Park, 2010).

Elaeodendron transvaalense ((Burtt Davy) R.H.Archer)

Botanical description

Elaeodendron transvaalense is a tree, which can sometimes reach a height of 10-15 m. The bark is utilized medicinally (Tshisikhawe and Van Rooyen, 2013). The flowers are greenish and they produce yellow to orange edible berries. The plant is widely distributed across the North-east parts of South Africa. It is also distributed along the coastal region of Kwa-Zulu Natal and Mpumalanga, Gauteng and Limpopo Province (Van Wyk *et al*., 2000). According to International Union for Conservation of Nature (2011) data list (IUCN) *E. transvaalense* is not threatened.

Other medicinal uses

An infusion of the bark is taken as a stomach cleanser and used as an enema for stomach aches, fever and to treat intestinal cramps and diarrhoea (Tshisikhawe and Van Rooyen, 2013). The leaves are chewed and the juice is swallowed for a sore throat (Van Wyk *et al.,* 2005). Stem bark is mostly used to treat coughs, herpes and venereal diseases (STDs) (Mabogo, 1990). It is also used in treatment of ulcers, fungal infections, piles and haemorrhoids in humans and domestic animals, and treatment of dysmenorrhoea (Bessong *et al.,* 2005).

Active components and pharmacological effects

Bark of *E. transvaalense* has been reported to have dysmenorrhoea in women (Steenkamp, 2003). The bioactivity for stomach ailments by *E. transvaalense* bark can at least be explained by the presence of phenolic compounds and tannins (Van Wyk *et al.,* 2005). This plant possesses anti-candida activity (Masevhe *et al*., 2015). Anti-cancer activity, Lup-20(30)-ene-3, 29-diol, lup-20(29)-ene-30-hydroxy-3-one and 4'-O-methylepigallocatechin are three pure flavonoids previously isolated from *E. transvaalense* (Mamba *et al*., 2016; kour, 2014). *E. transvaalense* has been found to have a low toxicity against vero and breast cancerous cells by Tshikalange and Hussein. (2010).

2.4. Conclusion

Five plant species have been documented for the treatment of both diabetes and arthritis, out of five plant species, three plants (*Bridelia mollis, Aloe micracantha* and *Elephantrorriza burkei*) were mentioned for the first time in treatment of diabetes. *Commiphora viminea, Elephantrorriza burkei* and *Senna petersiana* were also mentioned for the first time to treat arthritis. This shows that there is lots of work that still needs to be done in this area of diabetes and arthritis. This study has added value to an ever-increasing database of information relating to the medicinal value of the South African flora. Hence there is a need for a detailed investigation of ethnobotanical knowledge around the area before such vital knowledge vanishes into thin air. There is a huge gap that needs to be filled in the conservation and sustainable harvesting of medicinal plants.

Chapter 3: Anti-inflammatory activity of the selected medicinal plants used in the treatment of arthritis in Vhembe District Municipality, RSA.

Abstract:

Inflammation is one of the first responses to injury of living tissues; it has to be treated to avoid chronic inflammation. Overproduction of nitric oxide is one of the major causes of inflammation. The purpose of this study was to study anti-inflammatory action of five medicinal plants (*B. mollis, E. burkei, E. transvaalense, S. petersiana and S. lancea*) which are used traditionally to treat arthritis. Acetone, ethyl acetate, and ethanol extracts of five selected plants were tested for their antioxidants, anti-inflammatory and cytotoxicity, using DPPH, RAW 264.7 cells; and vero and bovine dermis cell lines for antioxidants, anti-inflammatory and cytotoxicity respectively. Cell viability was determined by mitochondrial reduction of 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) test. *Bridellia mollis* Hutch*,* and *Searsia lancea* (L.f.) F.A.Barkley had shown good antioxidant properties with LC_{50} lower than 0.5 µg/ml, and this was comparable to ascorbic acid and trolox. *Elephantorrhiza burkei* Benth, *Elaeodendron transvaalense (*Burtt Davy) R.H.Archer) and *Senna petersiana* (Bolle) Lock had shown poor antioxidant potential. All extracts showed a good NO inhibition, with highest percentage inhibition found in the highest concentration of 100 µg/ml; they all had a good percentage of cell viability at lowest concentration which was comparable to quercetin. Only two plant extracts *B. mollis* and *E. transvaalense* had lower than inhibition of quercetin at 25 µg/ml than at 12.5 µg/ml. In vero cells, low toxic effect was observed at lowest concentration tested, and toxicity increased with the increase in the concentration. In bovine dermis cell line all plant extracts had more cell viability at lower concentration than doxorubicin. The overall analysis showed that all plant extracts were toxic to both bovine dermis and vero cell lines. *S. lancea* was found to be the most toxic extract in this test. Further investigation of the plant species should include the use of aqueous plant extracts because local people use water as an extractant when preparing their herbal medicines.

Keywords: Medicinal plants; anti-inflammatory; antioxidant; RAW 264.7 cells; nitric oxide; cytotoxicity.

3.1 Introduction

Arthritis is one of the most common and prevalent form of arthritis worldwide (Fox and Stephens, 2010), and is one of the leading causes of pain and disability (Benjamin *et al.,* 2014). It affects every population and ethnic group, but most commonly elderly populations (Gabriel and Michaud, 2009). The World Health Organization estimated that globally, approximately 25% of adults aged over the age of 65 years suffer from pain and disability associated with this ailment (Breedveld, 2004).

The primary risk factors associated with arthritis are age, gender (more frequently in adult females), obesity, metabolic or endocrine diseases, trauma or joint overload, and also genetic factors (Cunha-Miranda *et al.,* 2015). Studies have not identified causal mechanisms responsible for the development of osteoarthritis, however, like any other acute and chronic inflammatory disease overproduction of nitric oxide is known to contribute to its development (Joo *et al*., 2014).

There are four main treatment categories for arthritis: non-pharmacological, pharmacological, complementary and alternative, and surgical (Keith *et al*., 2012). Unfortunately these treatments only bring down pain and improving the performance of the affected joints (Coman *et al*., 2012). Nonsteroidal anti-inflammatory drugs and selective Cyclo-Oxygenase-2 inhibitors, which are orally taken or applied, are recommended for patients who are not responding to acetaminophen, but unfortunately these medications can cause severe adverse side effects like gastrointestinal haemorrhage (Mulaudzi *et al.,* 2013).

Plants have formed the basis of sophisticated traditional medical systems that has been in existence for thousands of years, World Health Organization (WHO) estimated that about 80% of the world's population rely mainly on traditional medicines for primary health care (Yang *et al*., 2009). The latter is comparatively cheap, highly tolerated and convenient for many patients (Nagori *et al.,* 2010).

Inhibition of NO production in Lipopolysaccharide (LPS) stimulated RAW 264.7 cells is one of the possible ways to screen various anti-inflammatory drugs (Joo *et al*., 2014). Plants which are demonstrating inhibitory activities against NO production may have therapeutic potential for the treatment of inflammation accompanying overproduction of Nitric Oxide (Yang *et al.,* 2009). Since management of inflammatory diseases such as arthritis is difficult in developing and underdeveloped countries. This work was aimed at the evaluation of anti-inflammatory activity, antioxidant potential and cytotoxicity of selected medicinal plants used traditionally in the treatment of arthritis in the Vhembe District Municipality.

3.2 Methodology

3.2.1 Preparation of plant materials

Leaves of *Bridelia mollis* Hutch, *Elephantorrhiza burkei Benth, Senna petersiana* (Bolle) Lock, Searsia lancea (L.f.) F.A.Barkley and *Elaeodendron transvaalense (Burtt Davy) R.H.Archer* were collected in different areas of Vhembe District Municipality, Limpopo province in March, April and September 2015. In order to ensure the conservation of the medicinal plants, collection of leaves needs to be encouraged because collecting barks and roots threatens the survival of the plants. Hence, only leaves were gathered in this survey. Voucher specimens were prepared and deposited at the University of Venda herbarium for identification: *Senna petersiana* (Tp1)*; Elephantorrhiza burkei* (Tp2); *Bridelia mollis* (Tp3); *Searsia lancea* (Tp4); *Elaeodendron transvaalense* (Tp5). The leaves were dried at room temperature 25ºC for 15-21 days depending on the succulence of species, ground into powder using IKA-WERKE mill and stored at room temperature in airtight containers under dark conditions.

3.2.2. Plant extraction

Powdered leaves of (*B. mollis, E. burkei, S. petersiana*, *S. lancea* and *E. transvaalense*) were immersed into three different solvents (Acetone, Ethanol, and ethyl acetate). Powdered leaves were all left to steep in a covered container for 24 hours; the resulting infusion was decanted, filtered through a Büchner funnel and Whatman No.1 filter paper using a funnel, and evaporated using Büchi distillation chiller B-741 at 40°C. Dried plant extracts were weighed and dissolved in DMSO with a final concentration of 0.2%.

3.2.3 Phytochemical

Phytochemical compositions of the leaves of plant species were determined using the methods described by (Kazeem *et al.,* 2013).

Test for terpenoids

5 ml of each extract was added to 2 ml of chloroform and 3 ml of con. H_2SO_4 to form a monolayer of reddish brown coloration of the interface was shown to form positive result for the terpenoids (Sheikh *et al*., 2013).

Test for Saponins

The extract with 20 ml of distilled water was agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam indicated the presence of saponins (Sheikh *et al*., 2013).

Test for tannins

In the test for tannins, 0.5 g of dried, powdered sample was boiled in 20 ml of water in a test tube and filtered. A few drops of 0.1 % ferric chloride were added and observed for brownish green or a blue, black coloration (Kazeem *et al*., 2013).

Test for flavonoids

A portion of the powdered material was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. Development of yellow colouration is an indication of the presence of flavonoids (Kazeem *et al*., 2013).

Test for steroids

In this test, 2 ml of acetic anhydride was added to 0.5 g of extract with 2 ml concentrated H_2SO_4. The colour change from violet to blue or green is indication of steroids (Sheikh *et al*., 2013).

Test for anthraquinones

In the test tube 5 ml of chloroform was added to 0.5 g of the extracts of each specimen. The resulting mixture was shaken for 5 min, after which it was filtered. The filtrate was then shaken with equal volume of 10 % ammonia solution. The presence of a bright pink colour in the aqueous layer indicated the presence of anthraquinones (Kazeem *et al*., 2013).

Test for Cardiac glycoside

During the test 5 ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of the con. H_2SO_4. A brown ring of the interface indicated a deoxysugar characteristic of cardenolides. A violet ring might appear below the brown ring whereas acid layer, a greenish ring might form just gradually throughout thin Layer (Sheikh *et al*., 2013).

Test for alkaloids

Alkaloids were tested by the protocol used by Yusuf *et al.* (2014). The powdered leaves (2 g) were boiled in a water bath with 20 ml of 5% sulphuric acid in 50% ethanol. The mixture was cooled and filtered. A portion was reserved. Another portion of the filtrate was put in 100 ml of separating funnel and the solution was made alkaline by adding two drops of concentrated ammonia solution. Equal volumes of chloroform were added and shaken gently to allow the layer to differentiate. The lower chloroform layer was run away into a second separating funnel. The ammoniacal layer was reserved. The chloroform layer was extracted with two quantities each of 5 ml of dilute sulphuric acid. The various extracts were then used for the following test:

Dragendoff's test: To the filtrate in test tube II, 1 ml of dragendoff's reagent was added drop by drop. Establishment of a ruddy-brown precipitate indicates the presence of alkaloids.

3.2.4 Antioxidant assay (DPPH)

For Antioxidant (DPPH**)** assay, 40 µl of methanol was added to the first row of 96 well plates, 40 µl of the sample was placed in the first row of 96 well plates, serial dilution was done in all wells and last 40 µl was discarded. 160 µl DPPH was added to all the wells to make 200 µl in each well. Plates were left for 30 min, and then the absorbance was read at 517 nm. The experiment was performed in triplicate and average was considered.

3.2.5 Anti-inflammatory assay

3.2.5.1 Macrophages culture

RAW 264.7 macrophage cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Culture was carried out in a plastic culture flask in DMEM containing L-glutamine supplemented with 10% foetal calf serum (FCS) and 1% PSF (penicillin/streptomycin/fungizone) solution under 5% CO_2 at 37ºC. Cells were split twice a week. Cells were counted on a Neubauer chamber and suspension was prepared. The volume was determined using the formula below:

$$\text{Volume cells} = \frac{0.1 \times 10^6 \text{ cells/ml} \times 10 \text{ ml (for one plate)}}{\text{Initial conc cells/ml } (\times 10^6)}$$

3.2.5.2 Inhibition of nitric oxide (NO) production

To determine nitric oxide inhibition 100 µg/ml of the suspension containing 105 RAW 264.7 cells were introduced to 96 well-microtitre plates and Incubated for 24 h at 37ºC, 5% CO_2 to allow attachment. Macrophages were activated by incubation in a medium containing Lipopolysaccharide (LPS) (1 µg/ml) and simultaneously added the plant extracts of concentrations (100 µg/ml; 50 µg/ml; 25 µg/ml; 12, 5 µg/ml; 6,25 µg/ml and 3,125 µg/ml) and positive control (quercetin) of concentration (50 µg/ml; 25 µg/ml; 12,5 µg/ml; 6,25 µg/ml and 3,125 µg/ml) and then incubated for another 24 h at 37ºC, 5% CO2. 100 µl of the supernatant was collected and mixed with the same volume (100 µl) of Griess reagent and Incubated for 15 min in the dark. Finally the absorbance of the water-soluble purplish-red product was read on a BioTek Synergy microplate reader after 10 min at 550 (or 570) nm.

3.2.5.3 Interpretation of nitric oxide results

The nitric oxide amount was calculated using a calibration curve established by 0.15-100 µM of $NaNO_2$, percentage inhibition was calculated based on the ability of plant extracts or compounds to inhibit nitric oxide formation by macrophages compared to the control (cells in media without plant extracts or compounds containing triggering agents and DMSO).

$$\%\ \text{Inhibition} = \frac{\text{Absorbance (sample)}}{\text{Absorbance (Negative control)}} \times 100$$

IC_{50} values were calculated from the graph of percentage inhibition against different concentrations of the plant extracts or compounds.

3.2.5.4 Cytotoxicity assay (colorimetric MTT technique as described by Mosmann (1983))

African green monkey kidney (vero) and Bovine Dermis cells obtained from the American Type Culture Collection (Rockville, MD, USA), were maintained in Dulbecco`s modified eagle medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 1 % (PSF) penicillin/streptomycin/fungizone under standard cell culture conditions at 37 °C and 5 % CO_2 in a humidified environment.

One hundred (100) µl of a cell suspension of African green monkey kidney (Vero) or BD cells at a concentration of 100.000 cells/ml were seeded in a 96-well microtiter plate. Two hundred (200) µl of culture medium was added in columns 1 and 12 to minimize the "edge effect" and maintain humidity. Cells were incubated overnight (24 h) at 37ºC, 5% carbon dioxide (CO_2) to allow adhesion. 100 µl of plant extract or positive control (doxorubicin) added at different concentrations in each well of the microtiter plate extract and cells were diluted in small Eppendorf tubes considering the intensity required for each assay. (Note that the extracts were first diluted in DMSO and further dilutions were made with fresh medium). Cells were incubated for 2 days (48 hours) at 37°C, 5% (CO_2).

After incubation, MEM + extract or compound was removed from the wells, the latter was washed with 150 µl of PBS and 200 µl of fresh MEM was added to each well. Therefore 5 mg/ml MTT solution in PBS (0.015 g MTT in 3 ml PBS for the 1 plate) was prepared, and 30 µl of a 5 mg/ml MTT in PBS solution was added to each well, and the plates were incubated for a further 4 h at 37°C. After incubation, the MTT + MEM in the cells were taken out and rinsed with PBS (optional). MEM was carefully removed from each well, without disturbing the MTT crystals in the wells. MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. Plates were gently shaken until the solution was dissolved.

The amount of MTT reduction was measured immediately by measuring absorbance at reference wavelength of 570 nm and 630 nm. Wells in column 1, which contained medium and MTT but no cells, were used to blank the plate reader, to enable calculation of the percentage of cells in each well relative to the untreated control wells. The lethal concentration (LC_{50}) values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells at the concentration of 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml.

3.5 Results and Discussion

The purpose of the present work was to evaluate anti-inflammatory activity, antioxidant potential and cytotoxicity of ethanol, acetone and ethyl acetate extracts of *B. mollis* (T1)*, E. burkei* (T2)*, E. transvaalense* (T3)*, S. petersiana* (T4) *and S. lancea* (T5)*.* These plant species are utilized traditionally to manage arthritis in the Vhembe District Municipality, Limpopo Province, RSA.

3.5.1 Phytochemical analysis

Phytochemicals are plant-derived chemical compounds which are non-essential nutrients, some of which present potential health-promoting properties (Hussain *et al*., 2011). These secondary metabolites contribute significantly towards the biological actions of medicinal plants such as hypoglycaemia, anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antimalarial, anticholinergic, antileprosy activities (Yadav *et al*., 2014). For example, alkaloids protect against chronic diseases, saponins protect against hypercholesterolemia and have antibiotic properties (Mir *et al*., 2013).

Tannins speed up the healing of wounds and inflamed mucous membranes, flavonoids are powerful water-soluble antioxidants, which prevent oxidative cell damage, and they also assist in managing diabetes induced oxidative stress. Anthraquinones, tend to have laxative effects (Müller-Lissner, 1978). Terpenoids are useful in the prevention and therapy of various diseases such as malignant neoplastic diseases, and they possess anti-allergenic, antispasmodic, anti-hyperglycaemic, anti-inflammatory and immunomodulatory properties (Yadav *et al*., 2014).

Steroids are known to have a cardio tonic effect and also have antibacterial and insecticidal properties, cardiac glycosides have been employed to handle congestive heart failure and cardiac arrhythmia (Hussain *et al*., 2011). In this investigation phytochemical constituents of five different plants which are used medicinally in the Vhembe district municipality were evaluated.

Table 3 shows the presence of anthraquinones, saponins, flavonoids and tannins in acetone extract of *S. lancea* (TA5). Acetone extract of *S. petersiana* had saponins, flavonoids and cardiac glycoside. Acetone *E. burkei* (TA2) contained flavonoids and cardio glycosides. Saponins, steroids, flavonoids, anthraquinones and cardiac glycosides were also found in ethanol *E. transvaalense* (TE3). Bessong *et al*. (2005), have reported that there are saponins in in powdered roots of *E. transvaalense* (TE3). Ethanol *S. lancea* (TE5) contained all the compounds except terpenoids and cardiac glycoside.

This is in conflict with the study done by Gundidza *et al*. (2008), which have reported that antioxidant effect of *S. lancea* may be due to the monoterpenes. Ethanol *E. transvaalense* (TE3), *E. burkei* and *B. mollis* (TE1) had four phytochemical constituents, saponins, cardiac glycoside, steroids and flavonoids, and ethanol *B. mollis* (TE1) contained anthraquinones,

terpenoids and tannins, and ethanol *S. petersiana* (TE4) has been found to contain the least number of compounds, only flavonoids and cardiac glycoside. Flavonoid in *S. petersiana* (T4) is found in the study done by Mongalo, (2013). Ethyl acetate *B. mollis* (TEA1), ethyl acetate *E. burkei* (TEA2) contained only three phytochemicals each.

There is no work which showed phytoconstituents which have been found in these species. Ethyl acetate *E. transvaalense* (TEA3) possessed flavonoids, Tshikalange and Hussein. (2010), have found the presence of flavonoid compounds in this species. Ethyl acetate *B. mollis* (TEA1) had saponins, terpenoids and tannins, ethyl acetate *E*. *bukei* (TEA2) had saponins, cardiac glycoside and flavonoids, and ethyl acetate E. *burkei* (TEA2) had anthraquinones, cardiac glycoside and flavonoids as well. Mulaudzi *et al.* (2011), has reported that methanol root extract of *E. burkei* contained flavonoids and the latter have useful biological properties such as anti-inflammatory activity, enzyme inhibition and antimicrobial activity.

Ethyl acetate *S. petersiana* (TEA4) had four compounds steroids, cardiac glycoside, terpenoids and flavonoids. Out of all solvents which had been used to extract the compounds ethanol has extracted most compounds, 18 compounds in total. Maximum phytochemical constituents were obtained when extracting with ethanol. This agrees with the study conducted by Arya and jain. (2012). Terpenoids has been found in *B. mollis* (T1) and *E. transvaalense* (T3), and are known for their anti-inflammatory properties (Mahato and Sen, 1997). The presence of terpenoids support the use of *B. mollis* (T1) and *E. transvaalense* (T3) in traditional medicine for treatment of arthritis, which is an inflammatory disease.

Table 2. Phytochemical constituents of leaves of the selected plant species

Plant species	Phytochemical constituents								
	Extract	Anthraquinones	Saponins	Terpenoids	Cardio glycoside	Flavonoids	Tannins	Steroids	Alkaloids
B. mollis (T1)	Acetone	-	-	+	-	-	-	-	-
	Ethanol	+	-	+	-	-	+	-	-
	Ethyl acetate	-	+	+	-	-	+	-	+
E. burkei (T2)	Acetone	-	-	-	+	+	-	-	-
	Ethanol	-	+	-	+	+	-	-	-
	Ethyl acetate	-	+	-	+	+	-	-	+
E. transvaalense (T3)	Acetone	+	-	-	+	+	-	+	-
	Ethanol	-	+	+	+	+	-	+	-
	Ethyl acetate	+	-	-	+	+	-	-	+
S. petersiana (T4)	Acetone	-	+	-	+	+	-	-	-
	Ethanol	-	-	-	+	+	-	-	-
	Ethyl acetate	-	-	+	+	+	-	+	+
S. lancea (T5)	acetone	+	+	-	-	+	+	-	-
	ethanol	+	+	-	-	+	+	+	-
	Ethyl acetate	+	-	-	-	+	-	-	+

(+) = Presence, (-) = Absence

Qualitative phytochemical analysis of *B. mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S. lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts.

3.5.2 Antioxidant (DPPH) activity of the plant extracts

Antioxidants are interesting in the journey of drug discovery; several studies have shown a positive correlation between consumption of plant foods, which are rich sources of antioxidants and reduction of risk of diseases mediated by reactive oxygen species (Joo *et al*., 2014). Several antioxidant assays are frequently used to estimate antioxidant capacities in fresh fruits and vegetables, these assays can roughly be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET) ABTS and DPPH respectively (Bothon *et al*., 2013). In this study 2, 2- diphenyl-1-picrylhydrazyl (DPPH) which is ET assay was used to test for antioxidants.

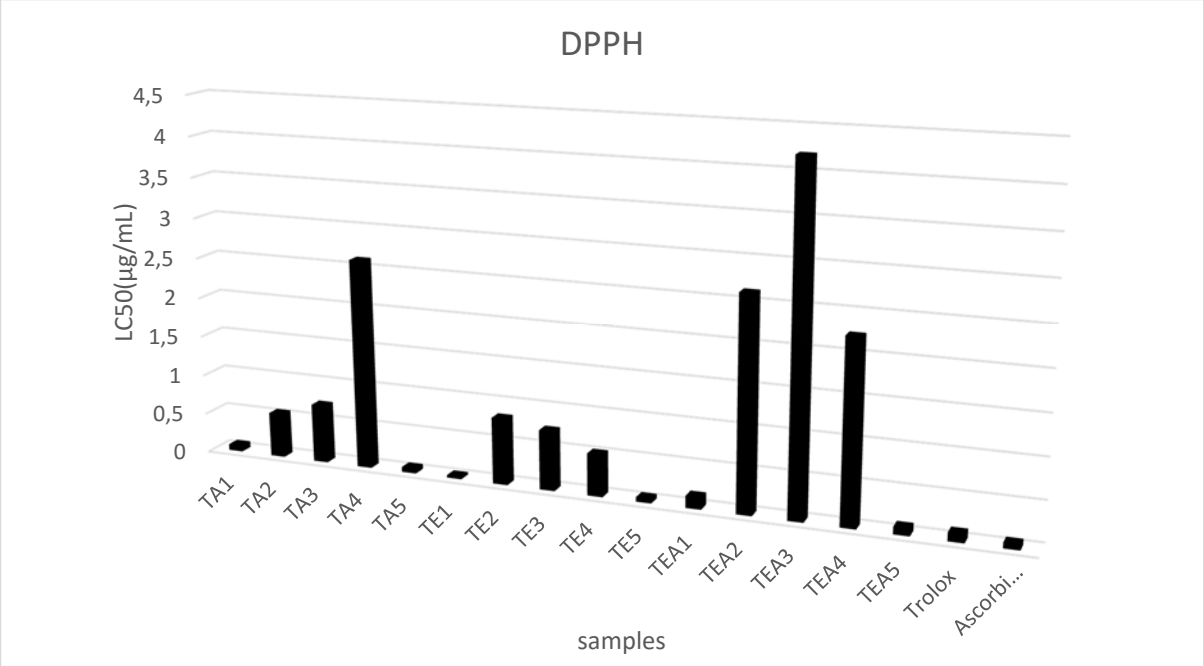


Figure 5. Antioxidant activities of *B. mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S. lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts using 2, 2- diphenyl-1-picrylhydrazyl (DPPH).

To determine the antioxidant potential of the plant extracts DPPH was used, ascorbic acid and trolox were used as positive controls as they are well known antioxidant supplements. Results obtained indicated that *E. burkei* (T2); *E. transvaalense* (T3*)* and *S. petersiana* (T4) had least antioxidant potential which was far below that of the positive controls, especially ethyl acetate extracts. Acetone and ethanol extracts of *E. burkei* (TA2), possessed antioxidant activities, and was due to the presence of phenolic compounds found in the species. Study by Tshikalange and Hussein. (2010), have reported that there is a correlation between phenolic compound and antioxidant content.

Ethanol was the best extractor for antioxidant screening as it had plants with LC_{50} which is lower than 1µg/ml in all five plants species. *B. mollis* (TE1) and *S. lancea* (TE5) extracts showed great antioxidants potential with all extracts, *B. mollis* (TE1) contained tannins, *E. transvaalense* (TA3*)* and *S. petersiana* (TA4) possessed flavonoids*. S. lancea* (TE5) had both flavonoids and tannins; they might be responsible for antioxidant potential in these two plant species. Bhandary *et al.* (2012), has reported that tannins are phenolic compounds; and plant phenolic are a major group of compounds that act as primary antioxidants or free radical scavengers.

In the study done by Gundidza *et al.* (2008), *S. lancea* essential oil showed antioxidant activity. The antioxidant potential found in essential oil might have been due to monoterpenes-pinene Bizuayehu *et al.* (2016), has reported that the total number of polyphenols, flavonoids and tannins positives correlate with the antioxidant activities. Antioxidant activities are found in the fruits of *B. mollis* in the work done by Ndhlala *et al.* (2006). There was a great difference in antioxidant potential found in acetone, ethanol and ethyl acetate extracts. According to Dutta *et al.* (2013), extraction solvent greatly influences the extraction of antioxidant components. *E. transvaalense* (TA3*)* and *S. petersiana* (TA4) contained flavonoids. It is reported that *S. lancea* possessed phenolics in ethyl acetate leave extracts in the study done by Hussain *et al*. (2011). These phenolic might be the one responsible for high antioxidant potential in these plant species, antioxidants are important because usually the healing properties of medicinal plants lie in the antioxidant properties (Ljubuncic *et al.,* 2005).

3.5.3 Nitric oxide inhibition by RAW 264.7 macrophages

Nitric oxide (NO) is an essential cytotoxic agent in host defence, but it can also be auto toxic if overproduced. Inducible NOS is responsible for excessive and auto toxic levels of NO in chronic pathogenic inflammatory lesions (Fang *et al*., 2010). NO production in Lipopolysaccharide (LPS) stimulated RAW 264.7 cells are one of the possible ways to screen various anti-inflammatory drugs (Joo *et al*., 2014). In the present study five plant species *B. mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) *and S. lancea* (T5) were evaluated for their NO inhibitory potential with their different extracts (acetone, ethyl acetate and ethanol).

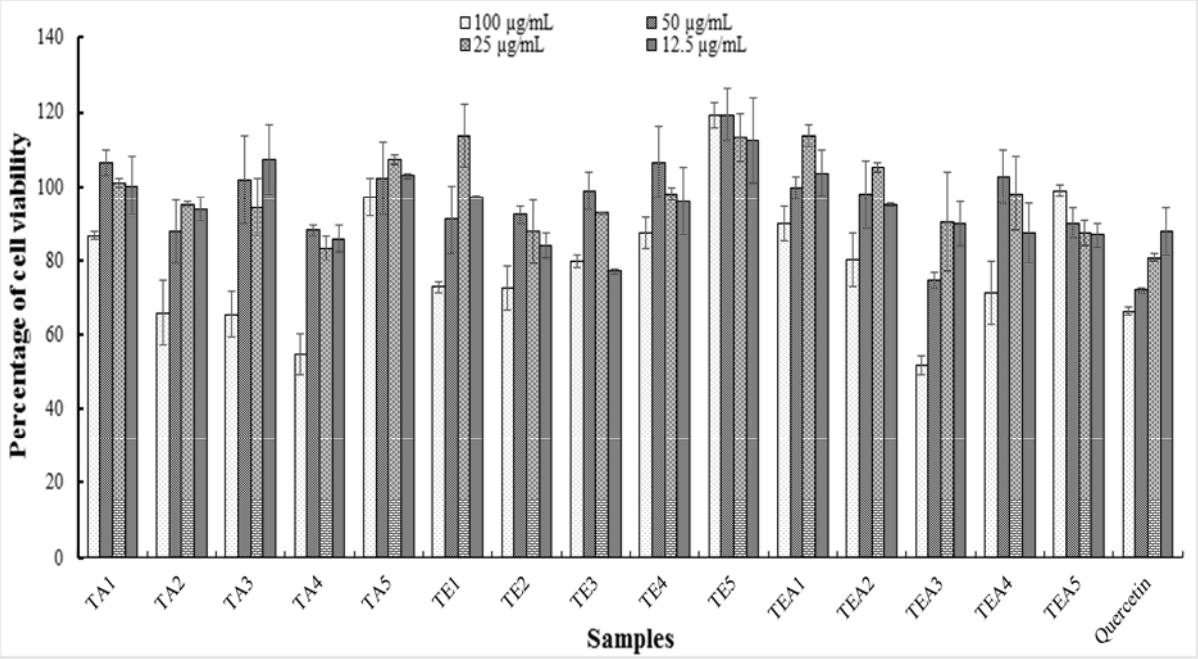


Figure 6 . Cytotoxicity of five plant species *B. mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S. lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts tested for their anti-inflammatory activity at different concentrations.

Almost all extracts showed the same pattern of cell inhibition, they were having few viable cells at highest concentration and more viable cells at lowest concentration except for ethyl acetate *S. lancea* which showed more viable cells at highest concentration and few viable cells at the lowest concentration (Figure 6). Jamalzadeh *et al*. (2016), it is found that the rate of cell viability decreased significantly when increasing concentrations of all examined plant extracts. All extracts had exhibited more than 60% cell viability at highest concentration (100 µg/ml) and more than 80% cell viability with (50 µg/ml, 25 µg/ml and 12.5 µg/ml) apart from ethyl acetate *E. transvaalense* (TEA3).

TEA3 showed high cytotoxicity towards the RAW 264.7 cell line compared to ethanol and acetone extracts. NO inhibition in ethanol *B. mollis* (TE1), acetone *E. burkei* (TA2) and ethyl acetate *E. burkei* (TA2) might be due to the presence of terpenoids (Table 3). It is reported by Wadood *et al.* (2013), that terpenoids exhibit various important pharmacological activities and anti-inflammatory is one them. In this study all plant extracts inhibited NO, this might be due to the presence of tannins and flavonoids. Acetone and ethanol *S.lancea* (TA5) extract showed the highest cell viability of more than 100%, this is in conflict with Gundidza *et al.* (2008), that has reported that *S. lancea* essential oil produces a resin which might be toxic. Inhibition of cells in all extracts were close to positive control at all concentrations or have more viable cells than the control, with only few having lower than that of the positive control (quercetin) at one or more concentrations. The number of viable activated macrophages was not significantly altered by the plant extracts as determined by MTT assays (Yang *et al*., 2009), this indicated that the inhibitory potential observed in these extracts was not simply due to toxicity of extracts to RAW 264.7 cells.

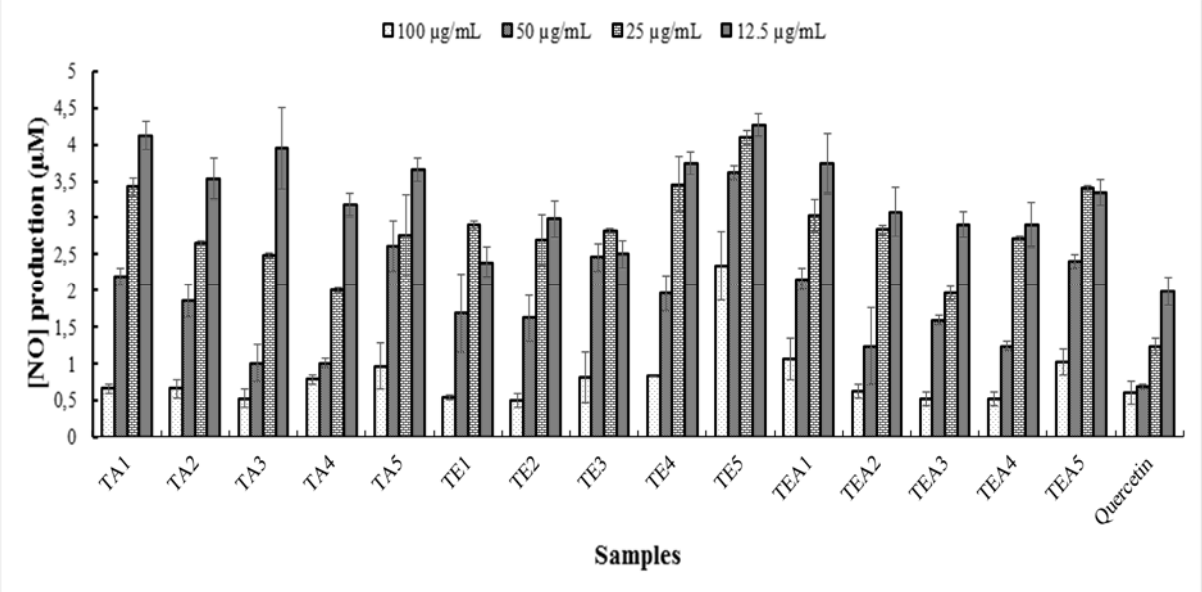


Figure 7. NO production by the RAW 264.7 cells in relation to different samples by five extracts of *B.mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S.lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts at different concentration in RAW 264.7 cells.

Reduction of NO production at inflammatory sites cause decreased production of peroxynitrite and reduced susceptibility to its tissue damaging effects (Levy and Simon, 2009). All plant extracts inhibited production of NO in a concentration dependent way, with the highest concentration having lowest NO production, and lowest concentration was having more NO production.

This was also observed in the study conducted by Ayele *et al.* (2013). Ethanol *B. mollis* (TE1), ethanol *E. transvaalense* (TE5) and ethyl acetate *S.lancea* (TEA5) were the only extracts which were showing higher NO production at 12.5 µg/ml than at 25 µg/ml. NO production of positive control was lower than in all extracts at 50 µg/ml, 25 µg/ml and 12.5 µg/ml respectively. This means that these plant extracts were not good candidates for anti-inflammatory drug production at a concentration of 50 µg/ml, 25 µg/ml and 12.5 µg/ml as they do not stop NO production in an efficient way like quercetin. At the concentration of 100 µg/ml the production of NO was the same or close to that of the positive control except for ethanol *S. lancea* (TE5) which had stopped NO production of 2.5 µM which was very high compared to all extracts as well as a positive control at that concentration. Ethyl acetate had lower production NO at the highest concentration of the plant extracts, and production was increasing with the decrease in concentration of the extract. Joo *et al*. (2014), found that ethyl acetate extract potentially inhibits the NO production in a concentration dependent manner. Looking at Fig 6 and 7 together helps to explain that Raw 264.7 cells were able to produce nitric oxide after activation with LPS. This is observed in an inhibition of NO Fig 8, it clearly indicate that the extracts had NO inhibitory potential.

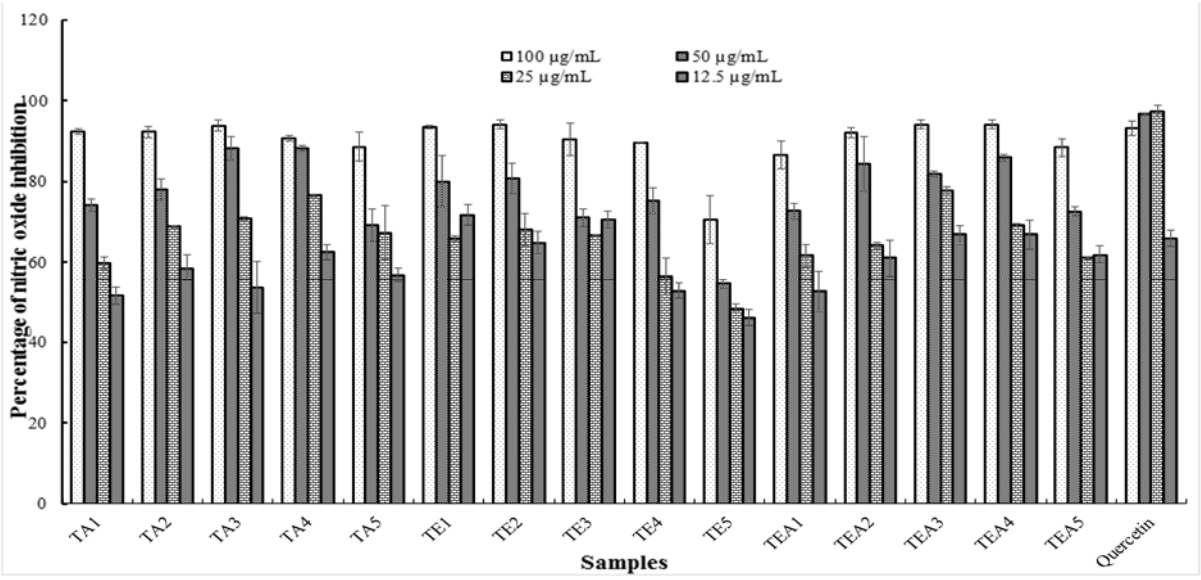


Figure 8. Indicates the percentage of NO inhibition by five plant species *B. mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S.lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts at different concentration in RAW 264.7 cells.

RAW 264.7 cell line has been utilized often to screen anti-inflammatory activities in plants for new drugs. The results in Fig 8 present percentage inhibition of NO by five plant species, (*B. mollis, E. burkei*, *E. transvaalense*, *S. petersiana and S. lancea*) and three extracting solvent (ethanol, Acetone and Ethyl acetate). Only two plant extracts *B. mollis* (T1) and *E. transvaalense* (T3) showed lowest inhibition at 25 µg/ml than in 12.5 µg/ml.

According to Yang *et al*. (2009), inhibitory effect of greater than 70% at 100 µg/ml is considered potent, so all extracts showed potent percentage inhibition at 100 µg/ml as they were having more than 80%. There was a very small difference in the inhibitory effects of NO and the solvents used for extraction, and there was a huge difference in the concentration of the sample and NO inhibitions, In the study done by Hyun *et al.* (2016), NO inhibition is found to be concentration dependent. All extracts had showed a good NO inhibition, with highest percentage inhibition found in the highest concentration tested, concentration was a major factor in the amount of NO inhibited by extract, and the increase in concentration the of plant extract caused an increase in inhibition of NO. The inhibition of NO in this study was comparable to the inhibition of quercetin drug. This support the use of these plants in the treatment of arthritis in traditional medicine.

3.5.4 Cytotoxicity assay (MTT technique) on Vero and bovine dermis cell lines

The ability of the cells to survive exposure to a toxic substance has been the foundation of cytotoxicity assays. This depends both on the number of viable cells and on the mitochondrial activity of cells. 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay is based on the theory that dead cells or their products do not reduce tetrazolium (Das and Devi, 2015). The non-cytotoxicity to the cells was expressed as the 50% non-toxic limit concentration ($NTLC_{50}$), which is the concentration of plant extracts to sustain the growth of cells up to 50% (Hanisa *et al.,* 2014). The LC_{50} of five plant species, namely *B. mollis, E. burkei, E. transvaalense, S. petersiana and S. lancea* in three solvents ranged from 1 mg/ml and 0.05 mg/ml.

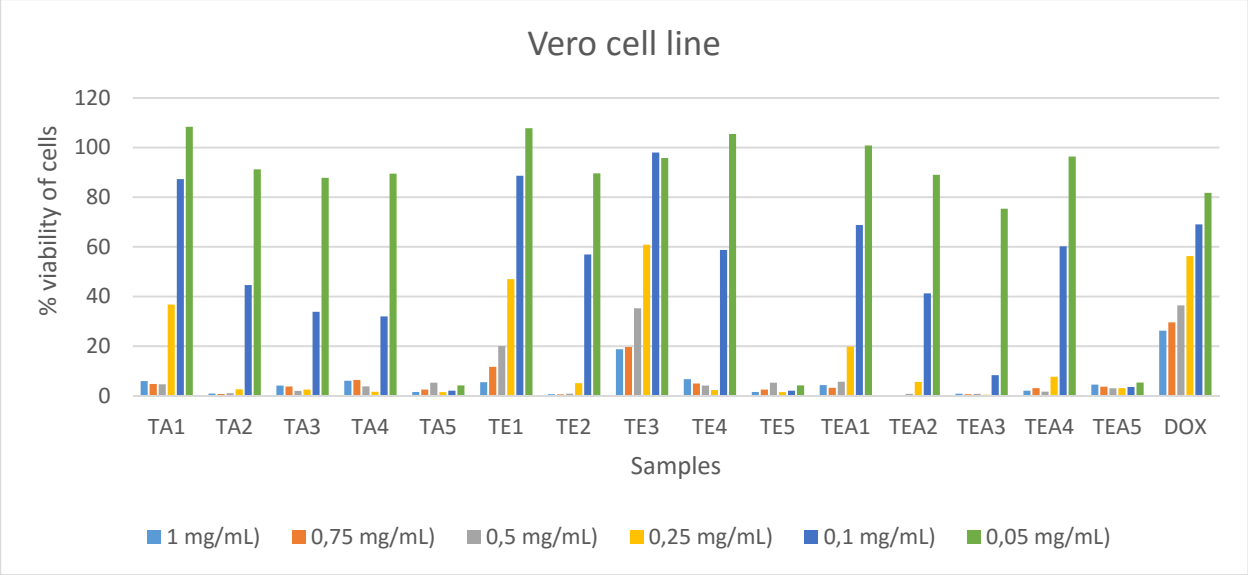


Figure 9 . Percentage viability of Vero cells after been treated with five different plant extracts *B.mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S.lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts at different concentrations.

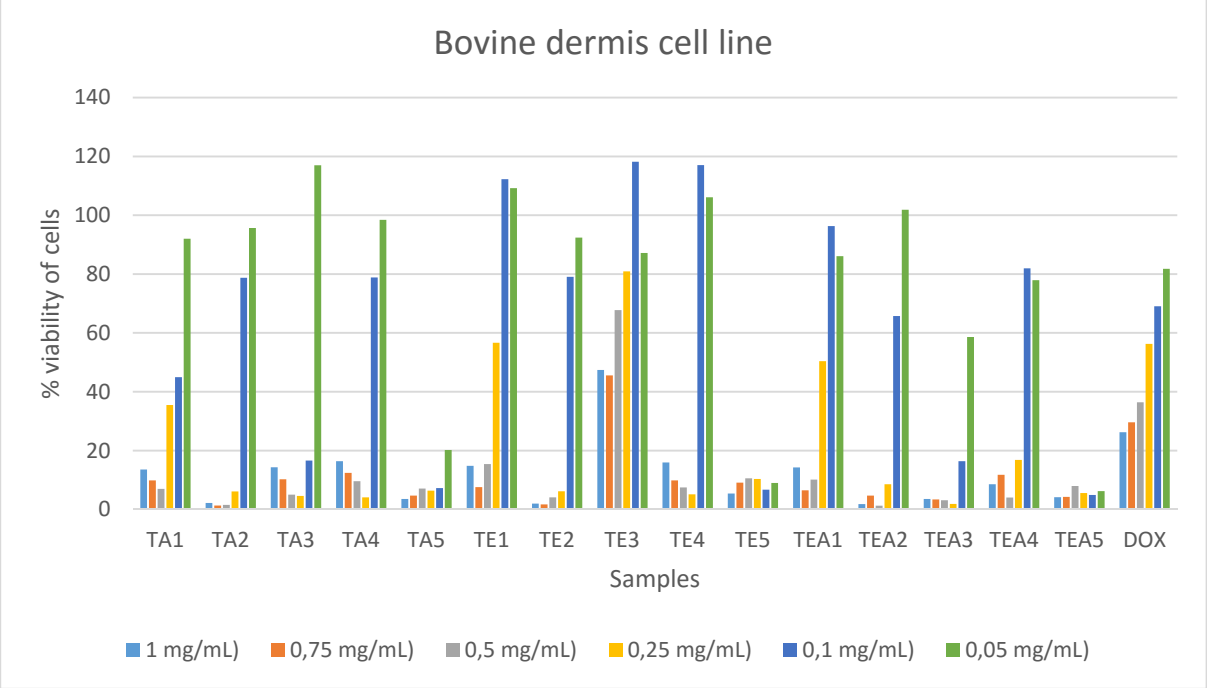


Figure 10. Percentage viability of Bovine dermis cells after been treated with five different plant extracts *B.mollis* (T1), *E. burkei* (T2), *E. transvaalense* (T3), *S. petersiana* (T4) and *S.lancea* (T5) with three different solvents E-Ethanol, A-Acetone and EA-Ethyl acetate extracts at different concentration.

All plant extracts shown in Fig 9 had a low toxicity effect against the Vero cell line at lowest concentration tested, and toxicity was increasing with the increase in the concentration, because at highest concentration there were least percentage of viable cells.

S. lancea (T5) has shown to have highest toxic potential with all extracts, though ethanol and acetone extracts of *S. lancea* (TA5) had moderate toxicity at 0.25 mg/ml and 0.05 mg/ml compared to ethyl acetate at the same concentration. High toxicity of *S. lancea* might be due to the presence of alkaloids and saponins. These compounds have been found to be toxic by Hanisa *et al.* 2014. Overall analysis has demonstrated that all plant extracts were toxic to bovine dermis and vero cell lines, this is indicated by less than 20% cell viability at one or more concentrations tested. Hanisa *et al*. (2014), stated that $NTLC_{50}$ is expressed as the minimal concentration that can support the growth of cells up to 50%. All plant extracts showed more cell viability at lower concentration than doxorubicin except for ethyl acetate *E. transvaalense* (TEA3) and *S. petersiana* (TEA4).

In Fig 10 ethanol extracts have shown less toxicity than all other extracts. *E. transvaalense* (TE3), *B. mollis* (TE1) were the only extracts more than 40% cell viability at 0.25 mg/ml. The overall cell viability of *E. transvaalense* was comparable to positive control doxorubicin. Ethyl acetate had shown the highest toxicity compared to acetone and ethanol, with acetone being the moderate one. *E. transvaalense* (TEA3), *S. lancea* (TEA5) *and E. burkei* (TEA2) high cytotoxicity might be due to the presence of alkaloids and saponins, “alkaloids are capable of specifically affecting the microtubule function and therefore blocking the establishment of the mitotic spindle” (Hanisa *et al*. 2014).

Ethanol leaves extracts *E. burkei (T2*) has been found to be non-toxic in the study performed by Mukanganyama *et al*. (2010), which differs with the result from this study. In this study *E. burkei* (T2) possessed saponins and according to Tshikalange and Hussein, (2010), saponins are toxic. This differs with the results from this study. *E. transvaalense* (T3) had been found to be toxic to both vero and bovine dermis ethanol and acetone extracts. It is found to be toxic to breast cancer in the study done by Kour. (2014), and vero cell line by Tshikalange and Hussein, (2010). All plant extracts showed high cell viability at the lowest concentration.

The overall analysis showed that more cell viability was observed in lowest concentration or second lowest concentration. In bovine dermis Fig 10 all plant extracts had exhibited more cell viability at lower concentration than the positive control, doxorubicin. Ethyl acetate *E. transvaalense* (TEA3) and *S. petersiana* (TEA4) were the only ones exhibiting less cell viability at the lowest concentration. Most extract showed more cell viability than doxorubicin in highest and second concentration. Ethyl acetate extracts had showed least number of cell viability with all extracts. In the study done by Tshikalange *et al. (*2005), *S. petersiana* (TEA4) showed significant toxicity to vaginal epithelial cell line.

There was an increase in the cell death at lower concentrations with bovine dermis. This was also observed by Harun *et al.* (2012), when ethanoic extract inhibited more MCF-7 cells over Vero cells at lower concentration. Most of Vero cells died at lowest concentration and survived the higher concentration on the study by Regina and Uma Rajan, *(*2014), Who also noticed that as the ethanoic extract concentration decreases the cytotoxicity effect increases in all the human and animal cell lines in *in vitro. S. lancea* had lowest cell viability in both Vero and bovine dermis cell line. At lowest concentration toxicity of all extracts was more outstanding than the toxicity of Doxorubicin at the highest concentration. In this study all extracts are toxic to vero and bovine dermis, all extracts except *E. transvaalense* (TEA3) had higher toxicity at 1 µg/ml, 0.75 µg/ml and 0.5 µg/ml than doxorubicin at the same concentration.

3.6 Conclusion

Bridellia mollis (TA1), *and Searsia lancea* (TA5) have shown a great antioxidant potential with all three extracts which is comparable to trolox and ascorbic acid, ethyl acetate showed least antioxidant with *Elaeodendron transvaalense* (TA3), *Senna petersiana* (TA4), these two plant species had least antioxidant activity with all extracts. All extracts were not lethal to the RAW 264.7 cell line. The inhibitory potential observed in these extracts is not due to their toxicity to RAW 264.7 cells after activation by LPS they were able to produce nitric oxide. This was a clear indication that the extract had the NO inhibitory potential. All extracts showed high percentage inhibition of NO at highest concentration which is comparable to that of the positive control (quercetin). Percentage inhibition of NO decreased with a decrease in concentration in all extracts. All extracts showed a huge toxicity towards vero and bovine dermis cell line, *S. lancea* was the most toxic plant species in all extracting solvents. The findings of this study indicated that all extracts deserve further investigation in order to isolate the bioactive metabolites which had anti-inflammatory properties and to conduct *in vivo* tests.

Chapter 4: *In vitro* Evaluation of alpha-amylase and alpha-glucosidase inhibitory properties of five plants from Vhembe district municipality, RSA

Abstract

Diabetes mellitus is a metabolic disorder in which the blood sugar level is higher than normal level either because the production of insulin is not enough or the cells do not properly respond to the insulin. Level of blood glucose in type-2 diabetes can be managed through inhibition of carbohydrate digesting enzymes, namely alpha-amylase and alpha-glucosidase. In this study, alpha-amylase and alpha-glucosidase inhibition activities of five plant species, *Bridellia mollis, Elephantorrhiza burkei, Elaeodendron transvaalense, Senna petersiana and Searsia lancea* used traditionally to manage diabetes were investigated using the standard *in vitro* procedures. The mode of action of the two enzymes was determined using Michaelis-Menten kinetics. The results showed that ethanol extracts of *B. mollis* and *S. petersiana*, and ethyl acetate extract of *E. transvaalense* had a good alpha-amylase inhibitory activity with IC_{50} values 58.6 and 81.9, 131.5 mg/ml respectively. On the other hand, hydro-ethanol, ethyl acetate and ethanol extracts of *E. burkei* exhibited a significant alpha-glucosidase inhibitory activity with IC_{50} values 56.9, 52.2 and 129.7 mg/ml, respectively while ethanol and hydro-ethanol extracts of *B. mollis* also had good inhibitory activity on the same enzyme with IC_{50} values of 64.4 and 84.4 mg/ml respectively. Only one aqueous extract of *E. burkei* possessed a considerable alpha-glucosidase inhibitory activity with IC_{50} value of 78.5 mg/ml. Kinetic analysis revealed non-competitive and un-competitive inhibitions of the plant extracts on alpha-amylase and alpha-glucosidase enzymes respectively. The observed good inhibitions of both alpha-amylase and alpha-glucosidase enzymes by plant extracts of *B. mollis, S. petersiana, E. transvaalense* and *E. burkei* validated their use in the traditional treatment of diabetes in the region.

Keywords: Medicinal plants; diabetes; alpha-glucosidase and alpha-amylase.

4.1 Introduction

Diabetes mellitus (DM) is a metabolic disease that has become a public health problem due to its serious long-term health complications (Coman *et al*., 2012). It is a group of metabolic disorders in which the blood sugar is higher than normal level either because the production of insulin is not enough (type 1 DM) or the cells do not properly respond to the insulin (type 2 DM) (Ndipa *et al.,* 2013). According to a report from the World Health Organization, about 220 million people have type 2 DM. Its incidence is increasing rapidly, and it is expected to increase to more than 365 million by 2030 (Nasri *et al.,* 2015).

Therapies which are currently available include insulin injection and oral anti-diabetic drugs such as acarbose, metformin, sulfonylurea, biguanides, inhibitors of alpha-glucosidase which are costly and disliked by patients because of their serious gastrointestinal side effects such as diarrhoea, gastritis, abdominal bloating (Patel *et al.,* 2012). As a result, there is an intensive interest in the scientific and medical communities for discovery of more efficient, safe and natural anti-diabetic drugs (Rahmatullah *et al.,* 2012).

Since ancient time medicinal plants and plant extracts have been used to combat a variety of diseases, including diabetes, especially in poor rural areas where the cost of conventional medicines is a burden to the people (Joseph and Jinni, 2013). Herbal medicines have been commonly prescribed throughout the world for several reasons, one of those reasons is that they have low to no side effects, they are easily accessible as well as affordable, (Bahmani *et al*., 2014), and they reduce the risk of diabetes and other diseases (Coman *et al*., 2012).

In a country like South Africa, where a great proportion of the population relies on plant-derived remedies to treat diseases, such inhibitors may come in the form of medicinal plant preparations (Shai *et al.*, 2010). This study is important as it will help with documentation of indigenous and traditional knowledge, as it is very crucial for future critical studies which will lead to sustainable utilization of natural resource (Singh *et al.,* 2012), thereby helping people realize the importance of maintaining biodiversity, the value of the plant species which surround their area as well as their medicinal uses. It is reported by Masevhe *et al*. (2015), that there is a need of detailed documentation on the use of medicinal plants in South Africa. Documentation of this knowledge can save lives of millions of people, benefits drug industry, and it can also increase the value of ethnomedicine and traditional pharmacology recognition in modern medicine.

The aim of this work was to evaluate anti-diabetic of medicinal plants used traditionally in the treatment of diabetes in the Vhembe District Municipality. The district municipality is one of the poorest in Limpopo Province and because of its rural nature and consequent high levels of unemployment and poverty; people in this area are heavily reliant on medicinal plants to treat several diseases (Semenya *et al*., 2012).

4.2 Materials and methods

4.2.1 Sample collection

Leaves of *Bridelia mollis* Hutch, *Elephantorrhiza burkei Benth, Senna petersiana* (Bolle) Lock, Searsia lancea (L.f.) F.A.Barkley and *Elaeodendron transvaalense (Burtt Davy) R.H.Archer* were collected in different areas of Vhembe District Municipality, Limpopo province in March, April and September 2015. In order to ensure the conservation of the medicinal plants collection of leaves needs to be encouraged because collecting barks and roots threatens the survival of the plants. Hence, only leaves were gathered in this survey. voucher specimens were prepared and deposited at University of Venda herbarium for identification: *Senna petersiana* (Tp1)*; Elephantorrhiza burkei* (Tp2); *Bridelia mollis* (Tp3); *Searsia lancea* (Tp4); *Elaeodendron transvaalense* (Tp5). Leaves were dried at room temperature 25ºC for 15-21 days depending on the succulence of species, ground into powder using IKA-WERKE mill and stored at room temperature in airtight containers under dark conditions.

4.2.2 Plant extraction

Plant materials from the five collected plants were extracted non-sequentially with 20 ml/g of aqueous, ethanol, hydro-ethanol and ethyl acetate by sonication for 1h each and left overnight with constant stirring using a magnetic stirrer. The temperature was kept lower by adding ice to the sonication bath. The extract was filtered through a Büchner funnel and Whatman No.1 filter paper. The concentrated extracts were then dried at room temperature under a stream of cold air and kept in air-tight containers until use. The dried material was weighed and resuspended in 10% dimethylsulfoxide (DMSO) to obtain a concentration of 50 mg/ml.

4.2.3.1 Alpha-Amylase inhibitory assay

The assay was carried out using a modified procedure of McCue and Shetty, (2004). 50 ml of each extract (62.5-1000 mg/ml) was pipetted into a test tube where 50µl of 0.02 M sodium phosphate buffer (pH 6.9) containing alpha-amylase solution was added. This solution was pre-incubated at 25°C for 10 min, after which 50 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and subsequently incubated at 25 °C for 10 min. The reaction was terminated by adding 100 µl of dinitro salicylic acid (DNS) reagent. The tubes were then incubated in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 1 ml distilled water and the absorbance was measured at 540 nm using a spectrophotometer (WPA Biowave II, Bichrom, England). The control was prepared using the same procedure replacing the extract with distilled water while the activity of the standard was tested by replacing the extract with acarbose (1.56-25 mg/ml). The alpha-amylase inhibitory activity was calculated as percentage inhibition following the expression below:

% Inhibition = [(Abs control-Abs extract)/Abs control] x 100

Where Abs is the absorbance reading of the samples and the concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

4.2.3.2 Mode of alpha-amylase inhibition

The method of Ali *et al*., (2006) was used to determine the mode of inhibition of the leaf extracts of five plant species (*B. mollis, E. burkei, E. transvaalense, S. petersiana* and *S. lancea*) and four extracts (aqueous, ethanol, hydro-ethanol and ethyl acetate). Using the extracts with the lowest IC_{50}. In brief, 250 µl of the extract (5 mg/ml) was pre-incubated with alpha-amylase solution (250 µl) for 10 min at 25ºC in one set of tubes. Therefore 250 µl of phosphate buffer (pH 6.9) was also pre-incubated with 250 µl of alpha-amylase solution in another set of tubes and starch solution (250 µl) of increasing concentrations (0.30-5.0 mg/ml) was added to both sets of reaction mixtures to start the reaction. The resulting mixture was then incubated for 10 min at 25°C and then suspended in a boiling water bath for 5 min after addition of 500 µl of DNS to stop the reaction.

A maltose standard curve was used to determine the amount of reducing sugars released and converted to reaction velocities. A double reciprocal plot (1/ [V] versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was plotted. The mode of inhibition of the extract on alpha-amylase activity was thereafter determined using Michaelis-Menten kinetics.

4.2.3.3 Alpha-Glucosidase inhibitory assay

The effect of the plant extracts on alpha-glucosidase activity was determined according to the method described by Kim *et al*. (2005), using an alpha-glucosidase from *Saccharomyces cerevisiae.* The substrate solution p-nitrophenyl glucopyranoside (pNPG) (5 mM) was prepared in 0.02 M phosphate buffer (pH 6.9). Briefly, 50 µl of the different concentrations of the extracts (1.56-25 mg/ml) was pre-incubated with 100 µl of alpha-glucosidase (0.5 mg/ml) in a test tube. Thereafter 50 µl of 5.0 mM (pNPG) as a substrate dissolved in 0.02 M phosphate buffer (pH 6.9) was afterward added to start the reaction. The reaction mixture was incubated at 37°C for 30 min and terminated by adding 2 ml of 0.1 M Na_2CO_3. The alpha-glucosidase activity was determined by measuring absorbance the yellow coloured para-nitrophenol released from pNPG at 405 nm. Percentage inhibition was calculated thus:

% Inhibition = [(Abs control-Abs extract)/Abs control] x 100

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

4.2.3.4 Mode of alpha-glucosidase inhibition

The modified method of Ali *et al*. (2006), was used to determine the mode of inhibition of the water extract with the lowest IC_{50} value. Briefly, 50 µl of the (5 mg/ml) extracts were pre-incubated with 100 µl of alpha-glucosidase solution for 10 min at 25ºC in five set of tubes and alpha-glucosidase was pre-incubated with 50 µl of phosphate buffer (pH 6.9) in another set of tubes. 50 µl of pNPG at increasing concentrations (0.25-2.0 mg/ml) was subsequently added to both sets of reaction mixtures to initiate the reaction. The resulting mixtures were then incubated for 10 min at 25°C, and 500 µl of Na_2CO_3 was added to terminate the reaction.

A p-μnitrophenol standard curve and converted to reaction velocities was used to determine spectrophotometrically the amount of reducing sugars released. A double reciprocal plot (1/V versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was plotted. The type (mode) of inhibition of the crude extract on alpha-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

4.3 Results and Discussion

4.3.1 Alpha-amylase and alpha-glucosidase inhibiting activity

Table 4 shows alpha-amylase and alpha-glucosidase inhibition activities of five *B. mollis, S. petersiana, E. transvaalense, E. burkei* and *S. lancea* medicinal plants used to treat diabetes in Limpopo Province, Vhembe District Municipality. Alpha-amylase is a glycoside hydrolase enzyme that breaks down long-chain carbohydrates (starch into maltose and dextrin), significantly decreases the digestion and uptake of carbohydrates, thereby decreasing the postprandial blood glucose level (Salehi *et al*., 2013).

Plant extracts having IC_{50} < 500 mg/ml are identified as the most potent plant inhibitors (*in vitro*) of digestive enzymes related to type-2 diabetes (Shori, 2015). Ethanol extracts of *B. mollis* and *S. petersiana,* and ethyl acetate extract of *E. transvaalense* showed noteworthy alpha-amylase inhibitory activity with IC_{50} values of 58.6 mg/ml, 81.9 and 131.5 mg/ml respectively. Ethanol has been reported to extract reducing sugar compounds in plants by Kazeem *et al*. (2013).

Hydro-ethanol and ethyl acetate extracts of *E. transvaalense* and *B. mollis* showed noticeable activity with IC_{50} values of 377.7 and 345.9 mg/ml respectively. *E. transvaalense* has been found to have positive alpha-glucosidase and alpha-amylase inhibition on the study done by Deutschländer *et al.* (2009). Aqueous plant extracts shows weak inhibitory effects on alpha-amylase. Overall, this study has shown a weaker alpha-amylase inhibition in all extracts except for Ethanol extracts of *B. mollis* and *S. petersiana* 58.6 and 81.9 mg/ml respectively. Alpha-glucosidase is an enzyme produced by the villi lining the small intestine of mammals and is responsible for the hydrolysis of disaccharides to monosaccharide that can be absorbed and consequently elevate blood glucose levels.

Inhibition of intestinal alpha-glucosidase has been used successfully to treat patients with both type I and type II diabetes mellitus (Deutschländer *et al.,* 2009). Aqueous and organic extract of *E. burkei* showed good inhibition activities ranging from IC_{50} values from 52.2 to 129.7 mg/ml. *B. mollis* exhibited strongest inhibition of alpha-glucosidase in ethanol and hydro-ethanol extracts with IC_{50} values ranging from 64.4 to 84.4 mg/ml (Table 4). This supports the traditional use of the plant extracts in the treatment of diabetes. This is attributed to the fact that plant phytochemicals are strong inhibitors of alpha-glucosidase and can be potentially used as an effective therapy for postprandial hyperglycemia with minimal side effects (Kazeem *et al*., 2013 (a)). It has been found by Mun'im *et al.* (2013) that plants which have strong alpha-glucosidase inhibitory activity generally contain alkaloids, tannins, terpenoids, saponnin, flavonoids and glycoside.

Literature has reported that in order for plant extracts to be good inhibitors they should not inhibit alpha-amylase more than alpha-glucosidase because excessive inhibition of pancreatic alpha-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon and that could lead to serious complications in the body systems (Olabiyi *et al.,* 2016). For example, aqueous extracts of *E. burkei* has shown a good inhibition of alpha-glucosidase of 78.5 mg/ml than the inhibition of alpha-amylase 1237.7 mg/ml (Table 4). Ethanol extracts of *B. mollis* showed good inhibitory effects on alpha-amylase and alpha-glucosidase with IC_{50} value of 58.6 and 64.4 mg/ml respectively. Ethanol and hydro-ethanol's ability to extract these inhibitory effects may be due to their polarity since they are really close to each other, so they are capable of extracting more or less the same inhibiting phytochemicals.

E. burkei and *B. mollis* hydro-ethanol extracts showed a good inhibition alpha-glucosidase 56.9 and 84.4 mg/ml respectively. Inhibition in these species could be due to phenolic compounds such as flavonoids and phenolic report by Zhang *et al.* (2015), that they have the ability to inhibit alpha-glucosidase. Although in this study out of these two plant species only *E. Burkei* was found to have flavonoids. Inhibition effects observed in ethanol and hydro-ethanol could be due to the fact that their polarity is very near to each other and they are capable of extracting more or less the same inhibiting phytochemicals. A good inhibition of ethanol bark extracts of these two enzymes has been reported in other studies (Gayathri and Jeyanthi, 2013).

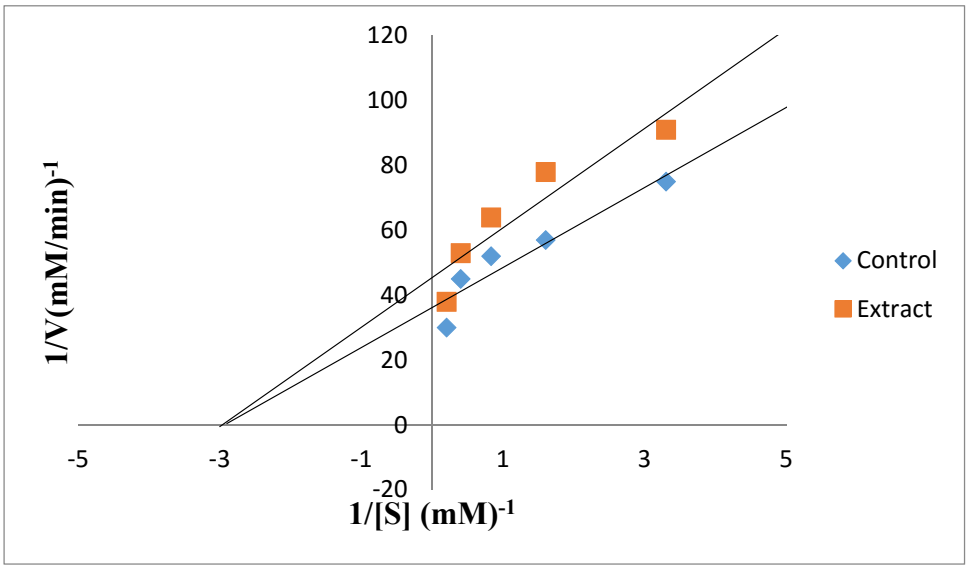
Hydro-alcoholic extracts have also shown inhibition of the same enzymes in the study done by (Sheliya *et al*., 2016). With all that has been found about these plants being good inhibitors of alpha-amylase and alpha-glucosidase, it does not mean that these plants can start to be used by humans, it is still too early to recommend them to be used because the *in vitro* inhibitory activity does not always relate to the corresponding in vivo activity (Samoa *et al.,* 2012). Thus, further tests need to be done in preclinical animal studies.

Table 4. IC_{50} (mg/ml) values alpha-amylase and alpha-glucosidase inhibition by leaf extracts of five plants which are used traditionally in the treatment of diabetes.

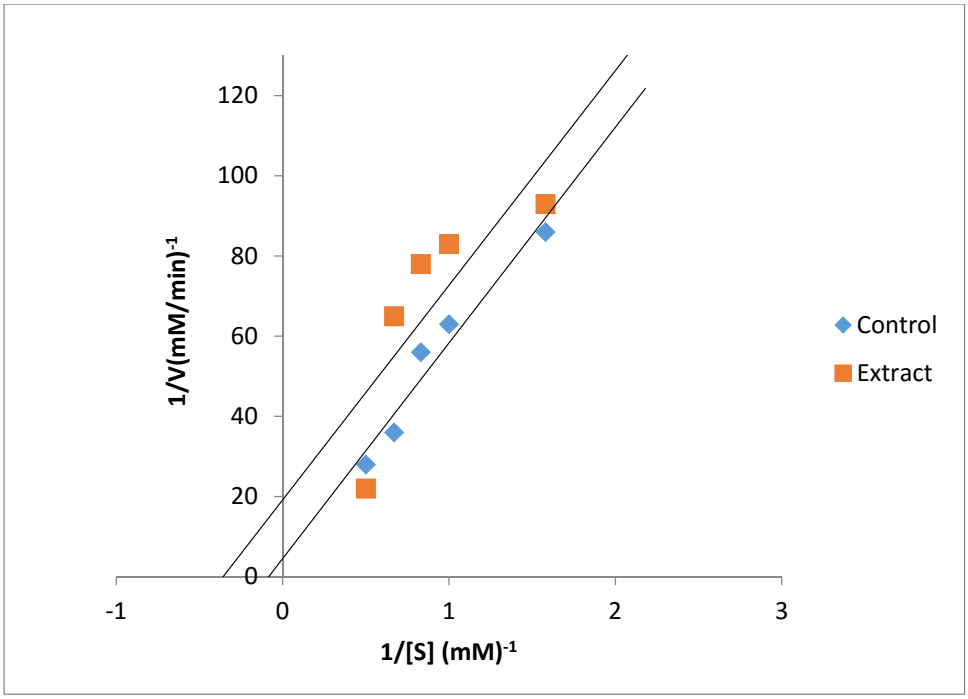
Plants species	Extracts	Inhibitory enzymes	
		Alpha-amylase IC_{50} (mg/ml)	Alpha-glucosidase IC_{50} (mg/ml)
B. mollis	Aqueous	974.7	268.4
	Ethanol	58.6	64.4
	Ethyl acetate	345.9	302.6
	Hydro-ethanol	987.0	84.4
E. burkei	Aqueous	1237.7	78.5
	Ethanol	653.7	129.7
	Ethyl acetate	829.0	52.2
	Hydro-ethanol	1996.4	56.9
E. transvaalense	Aqueous	597.4	365.8
	Ethanol	424.1	453.5
	Ethyl acetate	131.5	325.5
	Hydro-ethanol	377.7	371.1
S. petersiana	Aqueous	1661.9	594.1
	Ethanol	81.9	320.5
	Ethyl acetate	633.3	741.2
	Hydro-ethanol	688.4	429.4
S. lancea	Aqueous	658.3	550.7
	Ethanol	1008.6	507.0
	Ethyl acetate	443.3	546.4
	Hydro-ethanol	622.4	708.1

4.3.2 Kinetics of alpha-amylase and alpha-glucosidase

In this study lineweaver-Burk plot was used to determine the mode of inhibition of enzymes, alpha-amylase and alpha-glucosidase. *S. lancea* ethanoic extract Fig.11(a), *E. transvaalense* aqueous extract Fig.12(a) and Fig.12(b), *B. mollis* Fig.13(b) and *S. petersiana* hydro-ethanol extracts Fig.14(a) and Fig.14(b) have shown non-competitive inhibition against alpha-amylase and alpha-glucosidase activities. These observations indicated that the active component of the extract binds to a site other than the active site of the enzyme and combine with either free enzyme or enzyme substrate complex, possibly interfering with the action of both (Mayur *et al.,* 2010). Similarly, Lineweaver-Burk plot analysis revealed that *E. burkei* hydro-ethanol extract Fig.15(b) and *S. lancea* ethanoic extract Fig.11(a) had un-competitive inhibition against alpha-amylase and alpha-glucosidase activities. This suggests that the inhibitor binds exclusively to the enzyme-substrate complex yielding an inactive enzyme-substrate-inhibitor complex (Kazeem *et al.,* 2013(c)). Only one extract, *B. mollis* hydro-ethanol leaf extract Fig.13(a) inhibited alpha-amylase and alpha-glucosidase in a competitive manner. This mode of action suggests that the inhibitory component of the extract binds reversibly to the active site of the enzyme and occupies it in a mutually exclusive manner with the substrate (Kazeem and Ashafa, 2015).

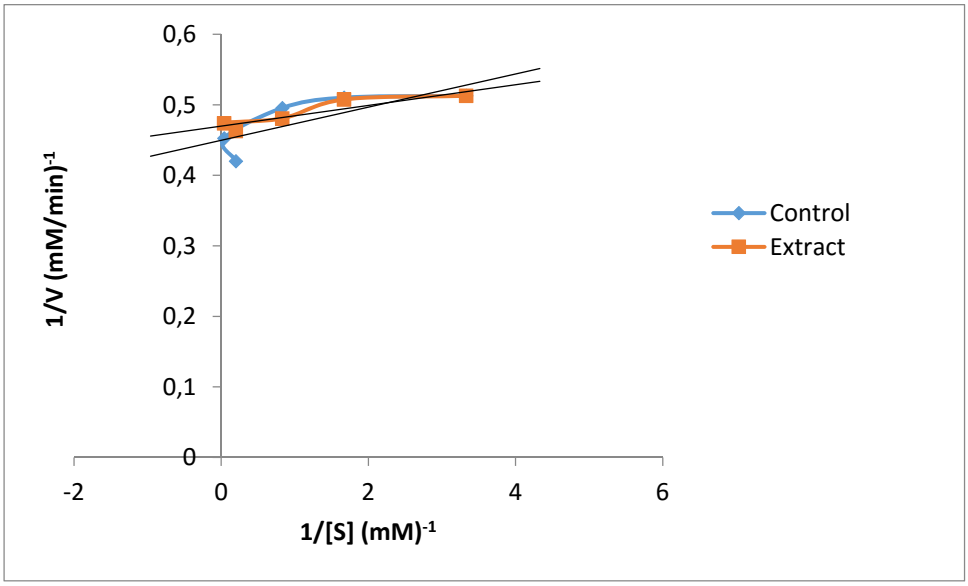


(a)

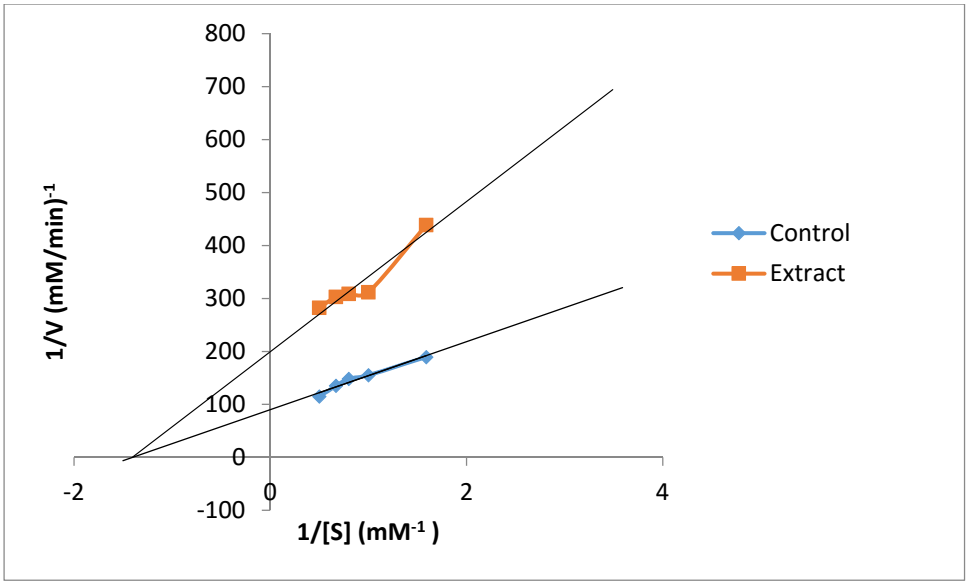


(b)

Figure 11: Lineweaver-Burk plot of *S. lancea* ethanoic leaf extract showing (a) non-competitive and (b) uncompetitive inhibition on alpha-amylase and alpha-glucosidase activities respectively.

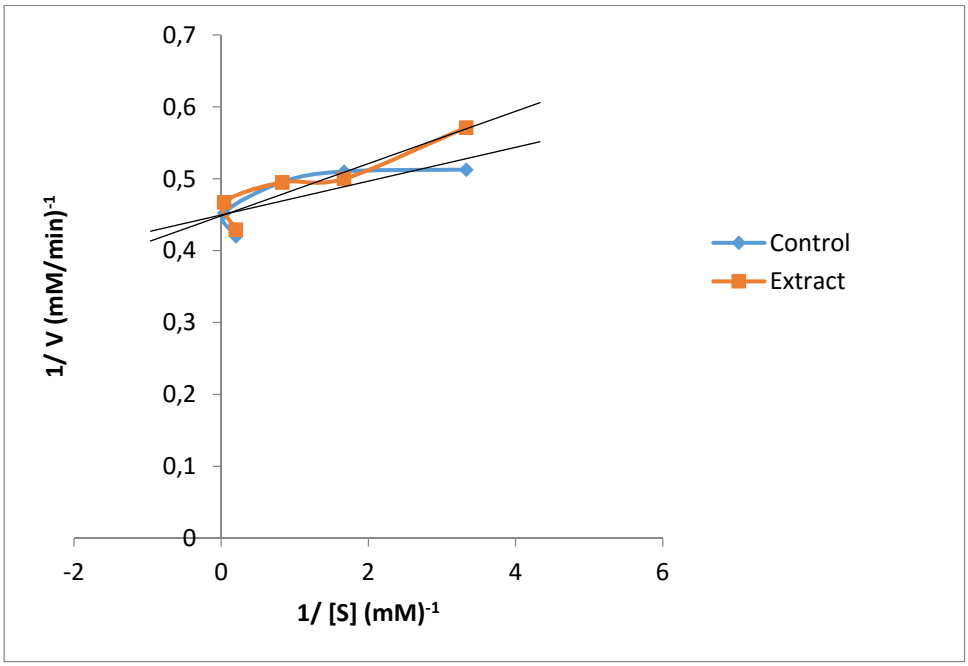


(a)

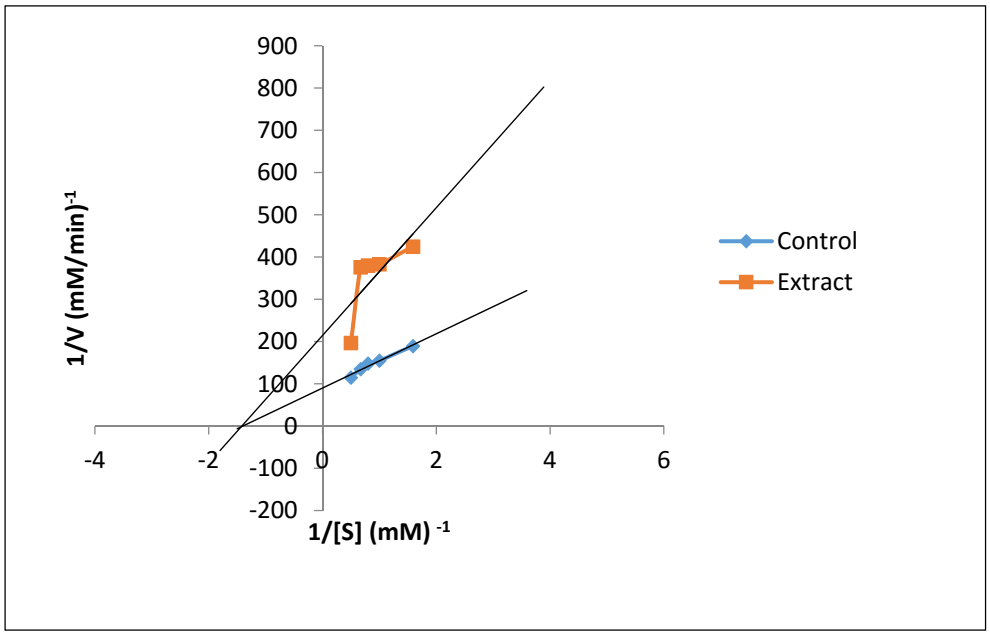


(b)

Figure 12: Lineweaver-Burk plot of *E. transvaalense* water leaf extract showing (a) un-competitive and (b) non-competitive inhibition on alpha-amylase and alpha-glucosidase activities respectively.

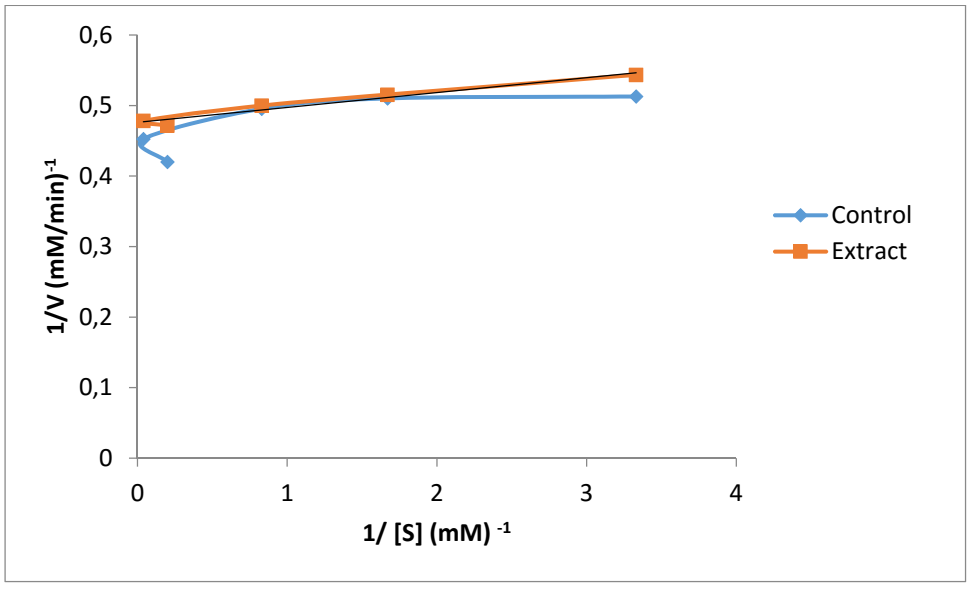


(a)

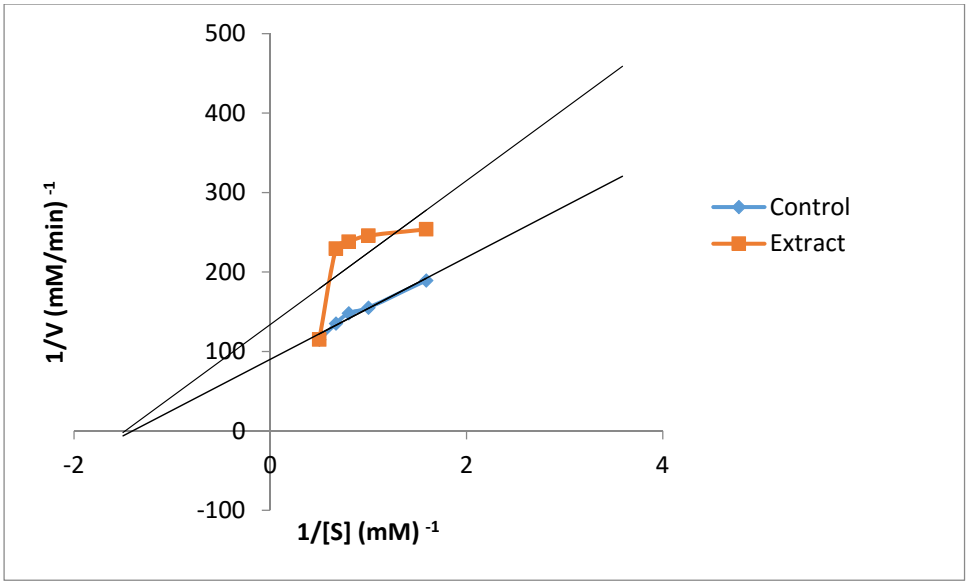


(b)

Figure 13: Lineweaver-Burk plot of *B. mollis* hydro-ethanol leaf extract showing (a) competitive and (b) non-competitive inhibition on alpha-amylase and alpha-glucosidase activities respectively.

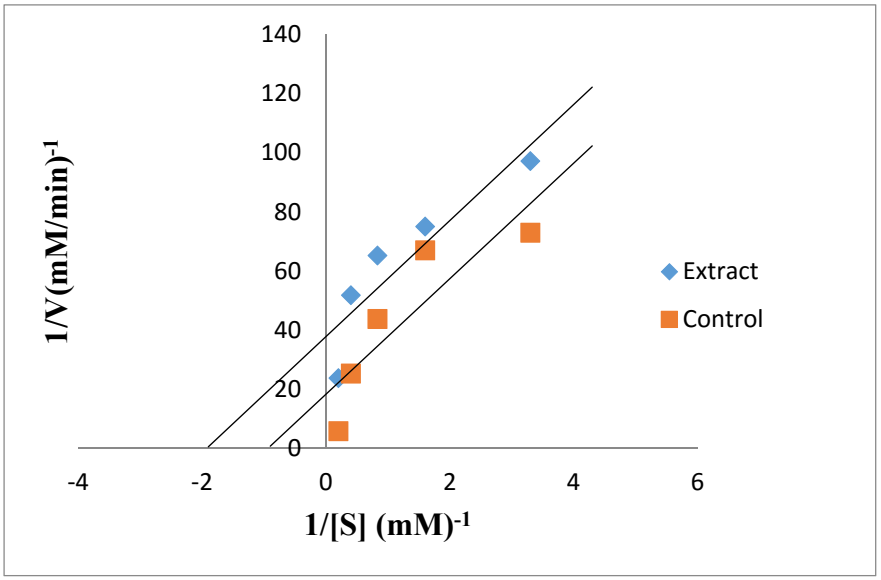


(a)

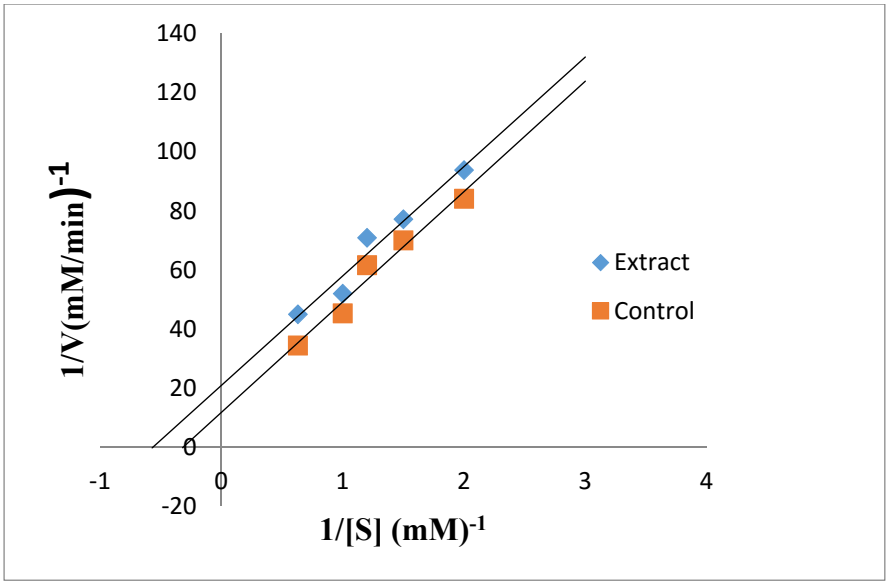


(b)

Figure 14: Lineweaver-Burk plot of *S. petersiana* hydro-ethanol leaf extract showing (a) uncompetitive and (b) non-competitive inhibition on alpha-amylase and alpha-glucosidase activities respectively.



(a)



(b)

Figure 15: Burk plot of *E. burkei* hydro-ethanol leaf extract showing uncompetitive inhibitions against (a) alpha-amylase and (b) alpha-glucosidase activity respectively.

4.4 Conclusion

It can be concluded that the observed good inhibitions of both alpha-amylase and alpha-glucosidase enzymes by extracts of *B. mollis, S. petersiana, E. transvaalense* and *E. burkei* support their use in the traditional treatment of diabetes by Vha-Venda people. The challenge for the rural communities is that they could not extract those phytoconstituents which are responsible for the enzymytic inhibitory effects because they use water. Interestingly, out of all the aqueous extracts investigated, aqueous extract of *E. burkei* had shown a potent anti-diabetic activity. The plant is commonly used by local people as decoction in the management of diabetes. Further *in vivo* work is needed to confirm the inhibitory activity of the plant extract.

4.5 Conflict of Interests

Authors declare no conflict of interest

Chapter 5: Discussion of main findings and conclusion

5.1 Introduction

Worldwide medicinal plants have been used to maintain health, managing diseases since human civilization, and they are continuing to supply people with remedies they need (Ramalivhana *et al.,* 2010). They have been used for treatment of diabetes and other complaints (Surya *et al.,* 2014). Most drugs which are used today have been produced from medicinal plants or at least first discovered in plants. Babajide *et al*. (2010), stated that since time immemorial plants have been a source of useful products such as lubricants, drugs, foods, gums and different additives. Therefore, exploring different patterns of plants which are known to contain phytochemical constituents is one way of making sure we protect humankind (Thang *et al.,* 2001). A great deal of study has been done on bioactivity of indigenous plants, which aid in the management several deadly illnesses which are serious health matters, but there is no much scientific work that has been performed in the most remote areas (Ahmed *et al.,* 2012). For all these reasons an ethnobotanical survey was conducted on medicinal plants which are used traditionally in the management of diabetes and arthritis and were also evaluated for their anti-diabetic and anti-inflammatory activity in order to validate their use in the traditional medicine.

5.2 Results and discussion

The study uncovered that only few people possessed knowledge about plants which are used for treatment and management of diabetes and arthritis. People above the age of 65 years were more informative than all age groups, women were more informative. The trees were found to be most commonly used, with leaves frequently used than all other parts of the plants. Most medicines used for both arthritis and diabetes were found to be prepared by decoction, and administered orally. For arthritis bathing and heat fumigation was mostly used, and orally administration common in diabetes. Some medicine combined with other during treatment which is common in traditional medicine. Five plant species have never been recorded in the treatment of diabetes (*Bridelia mollis, Aloe micracantha* and *Elephantrorriza burkei*) and three in the treatment of arthritis (*Commiphora viminea, Senna petersiana and Elephantrorriza burkei)*.

All plant extracts contained one or more phytochemical compounds, Ethanol has proven to be the best, extracting 18 compounds, *B. mollis* and *S. lancea* had beneficial antioxidant properties with LC_{50} lower than 0.5 µg/ml, and comparable to ascorbic acid and trolox with all solvents. Ethanol was the best extractor for antioxidants as it showed lowest LC_{50} with three plants which showed low antioxidants potential with other extractants. Ethyl acetate extracts had least of antioxidant potential, especially with *E. burkei, S. petersiana* and *S. lancea*.

All extracts showed a good NO inhibition, with highest percentage inhibition found in the highest concentration. They all had a high percentage of cell viability at the lowest concentration. Only two plant extracts, *B. mollis* (T2) and *E. transvaalense* (T3) showed lower inhibition. In vero cells, low toxic effect was observed at lower concentration, and toxicity increased with the increase in the concentration. In Bovine dermis all plant extracts had more cell viability at lower concentration than doxorubicin. Overall analysis showed that all plant extracts were toxic to both bovine dermis and vero cell lines, though *S. lancea* (T5) was found to be more toxic extract in this study.

Most extracts had few viable cells at highest concentration and more viable cells at lowest, with highest percentage inhibition in highest concentration, lowest inhibition at the lowest concentration. Percentage viability decreased with the decrease with concentration in all plant extracts, and all extract showed potent percentage cell viability. There was a very small difference in the inhibitory effects of NO and the solvents used for extraction. All acetone extracts had high toxicity effects on Vero cells. *S. lancea* at lowest concentration had toxicity which is higher than doxorubicin at the highest concentration; this was observed with all extracts. In ethyl acetate extracts, *S. lancea* was the only one with high toxicity. It was even higher than doxorubicin.

Ethanol extracts of *B. mollis* (TE1) and *S. petersiana* (TE4), and ethyl acetate extracts of *E. transvaalense* (TEA3*)* showed noteworthy alpha-amylase inhibitory activity. Interestingly, aqueous plant extracts of all five plant species showed weaker or very insignificant inhibitory effects on alpha-amylase. Aqueous extracts of *E. burkei* (T2) showed good alpha-glucosidase inhibition activities, followed by *B. mollis* (T2) which exhibited strongest inhibition with hydro-ethanol and ethanol extracts. This supports the traditional usage of the plant extracts in the management and treatment of diabetes. This study has shown that ethanol extracts had good inhibitory effects on both alpha-amylase and alpha glucosidase. E. *burkei* and *B. mollis* showed a good inhibition of alpha-glucosidase with hydro-ethanol.

Ethanol *S. lancea* extract (TE5), *E. transvaalense* aqueous extract (TE3) and *B. mollis* and *S. petersiana* hydro-ethanol extracts had showed non-competitive inhibition against alpha-amylase and alpha-glucosidase activities. Similarly, Lineweaver-Burk plot analysis revealed that *E. burkei* hydro-ethanol extract and *S. lancea* ethanoic extract (TE5) had un-competitive inhibition against alpha-amylase and alpha-glucosidase activities. Out of all extracts only one extract, *B. mollis* hydro-ethanol extract inhibited alpha-amylase and alpha-glucosidase in a competitive manner.

5.3 Conclusion and recommendation

The results have revealed that in the Vhembe District Municipality some people who still rely on medicinal plants for their primary health care. Results indicated that medicinal plants which are used in Vhembe district municipality do possess some bioactive compounds (antioxidants, anti-inflammatory and anti-diabetic) which aid in healing and maintenance of the health of local people. Some plant species were found to be toxic to the cells e.g. *S. lancea.* This research will help in documentation of medicinal plants used in the area; to add value to information related to the medicinal value of our flora and validate their use in traditional medicine. It has built a foundation for related studies in the area as it is the first study to combine anti-inflammatory and anti-diabetic. The aim of the study was achieved, plants used to treat diabetes and arthritis were documented as well as evaluating their *in vitro* efficacy. But due to few number of informants willing to share their knowledge few plants were documented and collected, and also because of lack of machinery and apparatus aqueous extract were only evaluated for anti-diabetics. Further survey is needed to investigate medicinal plants in the Vhembe District Municipality and its surrounding area to gather more ethnomedicinal knowledge. Furthermore investigation should include aqueous plant extract in anti-inflammatory, antioxidant and cytotoxicity studies since local people use water as an extractant when preparing their herbal medicines. Isolation of bioactive metabolites which are responsible for anti-diabetic and anti-inflammatory should be conducted.

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Appendix 1

UNIVERSITY OF VENDA, BOTANY DEPARTMENT

An inventory and pharmacological evaluation of medicinal plants used as anti-diabetes and anti-arthritis in Vhembe District Municipality, Limpopo Province, RSA.

Questionnaire

Informant's personal details

Name of the informant:

Physical address and contact telephone number

……

……

Male: Female:

Age:

Level of literacy:

Information about the disease

Do you treat individuals with diabetes/arthritis?

What are some of the symptoms related to diabetes/arthritis?

Do people who have been diagnosed with diabetes/arthritis at hospital/clinic come to you for treatment?

How many such persons are you currently treating?

Information about the medicinal plant spp. used

Family	Local name	Scientific name	Life form T, S, H, C	Parts used R, L, St, W	Availability VH, Hi, M, Lo, VL	Preparation mode	Ailment treated

Do you use a combination of plants when treating diabetes/arthritis patients? If yes, what are they?

Besides plant material(s) do you use other products like minerals or animal products? If yes, what are they……

Do you have any specific time/season for collection?.....

Can the plants be stored? If yes, what is your preservation method?

……

Information about the medicine

Preparation method:

Administration technique: Oral ☐ Smoke fumigation ☐ Heat ☐ fumigation ☐

Paste/poultice

Others

Dosage:

Do you have a dosage, if yes how much?

How long does the patient have to take the medicine?.....

Do you know any side effects of the plants you are using? Do you mention this to your patients?.....

Do you make any follow-up of your patients to see if they are fully recovered?

……

Comments:……