ANTIDIABETIC AND PHYTOCHEMICAL PROPERTIES OF FOUR SELECTED MEDICINAL PLANTS

By

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University of KwaZulu-Natal

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Sciences Higher Degrees Office for examination by the University appointed Examiners.

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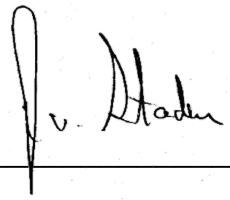

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DEDICATION

I would love to dedicate this work to the woman who made my coming to study at UKZN possible, My late grandmother, Mankwe Pricilla Mothapo. May your soul continue to rest in peace ‘koko Motšoadi’.

Blessed are they that mourn: for they shall be comforted.

(Mathew 5:4)

The LORD is my strength and my shield; my heart trusted in him, and I am helped: therefore, my heart greatly rejoiceth; and with my song will I praise him.

(Psalm 28:7)

Do not be anxious about anything, but in every situation, by prayer and petition, with thanksgiving, present your requests to God.

(Philippians 4:6)

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ABSTRACT

Diabetes mellitus (DM) or diabetes, a common metabolic disorder distinctively characterised by hyperglycemia, is a major non-communicable disease that affects millions of people globally, resulting in high incidences of morbidity and premature death rates. Chronic complications associated with DM include neuropathy, retinopathy, nephropathy and atherosclerosis. The management of diabetes has proven challenging especially in developing countries, due to costly ongoing treatments and often inaccessible health facilities. Due to the side effects and costs associated with current diabetes treatments, there is an upsurge in research into new antidiabetic agents specifically produced by plants.

In the present investigation four plant species, selected based on their traditional medicinal uses to treat diabetes, were subjected to a number of ethnopharmacological tests to validate their efficacy and to discover possible bioactive chemicals. Plant extracts were evaluated for their ability to inhibit enzymes involved in diabetes including α-amylase and α-glucosidase. Since oxidative stress and infections contribute greatly to complications related to diabetes, antioxidant and antimicrobial screening assays were performed and to further evaluate the safety of the plant extracts, mutagenic screenings were carried out. The extracts were also subjected to phytochemical analysis to evaluate the presence of secondary metabolites which are believed to form the basis of the bioactivities observed in plants.

The different plant specimens (*Bulbine natalensis, Kigelia africana, Leonotis leonurus* and *Sclerocarya birrea*) were collected from different locations; Mkhabathini farm KwaZulu-Natal, Ga-Mothapo village and the University of KwaZulu-Natal Botanical Garden, from March to April 2018. A voucher specimen for each species was deposited at the Herbarium of the University of KwaZulu-Natal, Pietermaritzburg. The dried plant materials were ground independently and extracted using different solvents of varying polarities (Petroleum ether,

Dichloromethane, Acetone and water). The resulting 24 extracts were evaluated for antidiabetic, antimicrobial and antioxidant activities. Fifty percent aqueous methanol extracts were subjected to phytochemical analysis.

The plant extracts were exposed to different free radical systems to observe their scavenging abilities. The extracts were subjected to *in vitro* heat induced β-Carotene oxidation, 1-1-diphenyl-1-picryhydrazyl (DPPH), hydrogen peroxide (H_2O_2) and ferric cyanide (Fe^{3+}) reducing antioxidant power (FRAP) screenings. The β-Carotene antioxidant activity was higher for ACE and water extracts of the stem bark of *S. birrea*. However, the PE extracts of both the leaf of *L. leonurus* and stem bark of *S. birrea* displayed no β-Carotene bleaching activity. The oxidation rate ratio was lower for ACE extract of the stem bark of *S. birrea* (0.05) than butylated hydroxyltoluene (BHT). The DPPH radical scavenging activity was higher in water and ACE leaf and root extracts of *B. natalensis*, DCM and water leaf extracts, ACE and water extracts of the fruit of *K. africana*, DCM, ACE and water extracts of leaves of *L. leonurus* and ACE and water extracts of *S. birrea* stem bark. The lowest IC_{50} was shown by ACE extract of fruit of *K. africana* and a water extract of the leaf of *L. leonurus* (0.01 mg/mL). The ability of the extracts to reduce H_2O_2 to water was better demonstrated by the ACE extract of roots of *B. natalensis*; water extract of fruit from *K. africana*, as well as ACE and water extracts of *S. birrea* stem bark. The ferric reducing power was better demonstrated by a PE extract of the fruit of *K. africana*; DCM leaf extract of *L. leonurus* and also ACE and water extracts of *S. birrea* stem bark. Extracts exhibiting weakest reducing power at the highest concentrations tested (12.5 mg/mL) were PE and DCM extracts of *S. birrea* stem bark; ACE leaf extract of *K. africana* and water extract of leaves of *B. natalensis*.

Preliminary phytochemical screening revealed the presence of flavonoids, alkaloids and carbohydrates for all plant extracts. The absence of certain classes of secondary metabolites during screening were observed in leaves of *B. natalensis* (phenols, terpenoids, tannins and

steroids), fruit of *K. africana* (phenols and steroid) and leaves of *K. africana* (steroids). The spectrophotometric phytochemical analysis revealed the quantities of some secondary metabolites (total phenolic content, flavonoids and condensed tannins) in the plant species. The stem bark of S. *birrea* exhibited the highest total phenolic content (41,21 mg GAE/g), flavonoids (175,78 mg CE/g) and condensed tannins (1,91 mg CCE/g). Roots of *B. natalensis* showed the lowest phenolic content (5,31 mg GAE/g) and leaves of *K. africana* had low levels of both flavonoids and condensed tannins (30,08 mg CE/g and 0.14 mg CCE/g, respectively).

The antidiabetic inhibitory screening of the α-amylase and α-glucosidase enzymes revealed the potentials of the different plant extracts to act as natural antidiabetic agents. A modified microtitre plate technique was used to evaluate the α-glucosidase inhibitory activity, while a modified DNSA (3.5-dinitrosalicyclic acid solution) method was used to evaluate the α-amylase inhibitory activity of the plant extracts at 100 µg/mL. High α-amylase inhibitory activity was observed in DCM and water extracts of leaf extracts of *B. natalensis*, ACE extract of leaves of *L. leonurus* and water extracts of leaves of *K. africana* and *S. birrea* stem bark. Potent α-glucosidase inhibitory activity was shown by DCM and ACE extracts of roots of *B. natalensis*, ACE extracts of leaves of *B. natalensis*, ACE extracts of fruit of *K. africana* and ACE and water extracts of *S. birrea* stem bark. Better antidiabetic activities were exhibited by the organic extracts as compared to the water extracts.

For antimicrobial screening, test organisms from the American Type Culture Collection (ATCC) were used to evaluate the ability of plant extracts to inhibit pathogenic bacteria. Gram-positive (*Enterococcus faecalis*, ATCC 19433 and *Staphylococcus aureus*, ATCC 12600) and Gram-negative (*Escherichia coli*, ATCC 11775 and *Klebsiella pneumoniae*, ATCC 13883) and the yeast *Candida albicans* (ATCC 10231) strains were used for this investigation. The microplate dilution technique was adopted to determine the minimum inhibitory concentration (MIC) of the extracts. High antimicrobial inhibitory activity against both the antibacterial and

antifungal strains was demonstrated by the DCM extract of roots of *B. natalensis*, ACE and water extract of *S. birrea* stem bark and ACE extracts of leaves of *L. leonurus* (MIC < 1 mg/mL). However, the DCM extract of leaves of *B. natalensis* and the ACE extract of roots of *B. natalensis* did not display effective microbial inhibitory activity against all bacterial strains tested (MIC > 1 mg/mL).

The plant extracts demonstrating complimentary antidiabetic inhibitory activities against α-amylase and α-glucosidase, were selected and further subjected to the Ames test using tester organisms (TA 102 and 1535) without the S9 metabolic activation, to evaluate their safety. All tested extracts did not display mutagenicity toward the tester organisms. However, the ACE extract of the stem bark of *S. birrea* displayed some potential to become mutagenic. These findings emphasise the importance of investigating the safety of natural products consumed for medicinal purposes. The obtained information further validates the traditional use of these medicinal plants as antidiabetic medicines and provides a valuable opportunity to discover potential sources of novel treatments against the carbohydrate hydrolysing enzymes to alleviate or prevent DM.

CONFERENCE CONTRIBUTION FROM THIS THESIS

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LIST OF ABBREVIATIONS

4NQO - 4-nitroquinoline–N-oxide

A1C - Airman First Class

ACE - Acetone

AIDS - Acquired Immunodeficiency Syndrome

ATCC - American Type Culture Collection

ASC - Ascorbic Acid

BCA- β-Carotene/linoleic Acid

BHA - Butylated Hydroxylanisole

BHT - Butylated Hydroxyltoluene

CAM - Complementary Alternative Medicine

CAT - Catalase

CCE - Cyanidin Chloride Equivalent

CE - Catechin Equivalent

CFU - Colony Forming Units

CVD - Cardiovascular Disease

DCCT - Diabetes Control And Complications Trial

DCM - Dichloromethane

DKA - Diabetic Ketoacidosis

DM - Diabetes Mellitus

DMSO - Dimethyl Sulphoxide

DNSA - 3.5-Dinitrosalicylic Acid

DPPH - 2,2-Diphenyl-1-picryl Hydrazyl

DW - Dry Weight

EC - Effective Concentration

FRAP - Ferric Cyanide (Fe3+) Reducing Antioxidant Power

GAE - Gallic Acid Equivalent

GBDS - Global Burden of Diseases Study

GDM - Gestational Diabetes Mellitus

H_2O_2 - Hydrogen Peroxide

HbA1c - Haemoglobin A1c

HIV - Human Acquired Immune Deficiency

IC - Inhibitory Concentration

IDDM - Insulin Dependent Diabetes Mellitus

IDF - International Diabetes Federation

IEC - International Expert Committee

INT - Iodonitrotetrazolium Chloride

MH - Mueller-Hinton

MIC - Minimum Inhibitory Concentration

NCD - Non-Communicable Disease

NDDG - National Diabetes Data Group

NGSP - National Glycohaemoglobin Standardization Program

NIDDM - Non-Insulin Dependent Diabetes Mellitus

OGTT - Oral Glucose Tolerance Test

ORR - Oxidation Rate Ratio

PE - Petroleum Ether

PG - Plasma Glucose

PPB - Potassium Phosphate Buffer

ROS - Reactive Oxidation Species

RSA - Radical Scavenging Activity

SPB - Sodium Phosphate Buffer

SSA - Sub-Saharan Africa

SSB - Sugar-Sweet Beverages

STZ - Streptozotocin

T1DM - Type 1 Diabetes Mellitus

T2DM - Type 2 Diabetes Mellitus

TB - Tuberculosis

TZD - Thiazolidinediones

UKZN - University of Kwazulu-Natal

UTI - Urinary Tract Infection

WHO - World Health Organisation

YM - Yeast malt

α - Alpha

β - Beta

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Humanity has always been susceptible to diseases caused by pathogens or as a consequence of aging **(ARMELAGOS et al., 1996)**. To date, communicable and non-communicable diseases have had devastating impacts worldwide. Communicable diseases are responsible for high levels of morbidity and mortality globally **(SAKER et al., 2004; WHO, 2010)**. For example, the global burden of disease study (GBDS) reported that in 2002, communicable diseases contributed to approximately 52% of deaths in developing countries **(WHO, 2008)**. The epidemics and pandemics of communicable diseases have caused overwhelming health and socio-economic consequences especially in low income countries where the burden of these diseases is higher than in other countries **(SAKER et al., 2004; WHO, 2010)**. However, amid all these health issues, non-communicable diseases (NCDs) are now competing with communicable diseases, adding to morbidity and premature mortality rates higher than those caused by infectious diseases **(SAKER et al., 2004)**. The burden of non-communicable diseases can be catastrophic in developing countries, where there are higher incidents of communicable diseases, limited health facilities in addition to exhausted health budgets and poverty **(MAYOSI et al., 2009; BOUTAYEB and BOUTAYEB, 2005)**. Alarmingly, according to the World Health Organisation (WHO), in South Africa the burden of non-communicable diseases is three-fold higher than in developed countries **(MAYOSI et al., 2009)**.

1.2 Non-communicable diseases (NCDs)

Non-communicable diseases (NCDs) are not only a worldwide burden but are a major public health concern, challenging social and economic development globally **(WHO, 2018)**. There are several causes of NCDs including genetics, environmental and behavioural constituents **(WHO, 2018)**. Due to the persistent nature of these diseases, they are classified as chronic diseases. Major types of NCDs include cardiovascular diseases (CVDs), cancers, chronic respiratory diseases and diabetes mellitus **(WHO, 2018)**. NCDs are the primary cause of premature mortality globally and account for over 80% of deaths in low and middle-income countries **(MISGANAW et al., 2014; WHO, 2018)**.

At present, the population of the African continent is predominantly affected by infectious diseases such as Human immunodeficiency virus/ Acquired immunodeficiency syndrome (HIV/AIDS) and Tuberculosis (TB) **(WHO, 2018)**. However, the World Health Organisation (WHO) has suggested that by the year 2030, NCDs will become major causes of death in the region **(WHO, 2018)**. For example, in South Africa the Burden of Disease Research Unit at the South African Medical Research Council reported that NCDs are the leading cause of death in South Africa (SA) surpassing the rates of mortality caused by HIV/AIDS and TB combined **(NOJILANA et al., 2016; WHO 2013)**.

1.3 Diabetes mellitus

Diabetes mellitus (DM) is considered to be one of the four major non-communicable diseases worldwide **(WHO, 2016)**. DM is a metabolic disorder that results from a deficiency in the production and/or utilisation of insulin **(MARITIM et al., 2003)**. Hyperglycemia, an increase in blood glucose levels, is a common trait of diabetes and causes a disruption in functioning of proteins, fats and carbohydrates **(BILAL et al., 2018; GOBOZA et al., 2016)**. DM is a global concern affecting all population classes and races, and as such, has become one of the most

important global health challenges **(BILAL et al., 2018; WHO, 2016)**. Diabetes occurs in either of three forms; Type 1; Insulin Dependent Diabetes Mellitus (IDDM) resulting from a complete deficiency of insulin caused by the immunological destruction of the pancreatic β-cells. Type 2; Non-Insulin Dependent Diabetes Mellitus (NIDDM) triggered by limited insulin production from the pancreatic β-cells **(GOBOZA et al., 2016)**. Type 3; gestational diabetes which occurs during pregnancy because of dysglycemia **(McDONALD, 2008)**.

Generally, DM is initiated by hypoglycemic conditions in the body and insulin resistance by targeted cells. DM is one of the apparent causes of increased morbidity and mortality worldwide **(GOBOZA et al., 2016; WHO, 2016)**. The development of DM can also be attributed to external factors such as environmental conditions and the intake of certain food products that can lead to the mutation of immunological cells through the destruction of β-cells **(HNATYSZYN et al., 2002; CUEVAS et al., 2008)**. The use of steroids can also trigger the development of diabetes **(FERNER, 1992)**.

1.3.1 Classification and pathogenesis of Diabetes mellitus

A classification system is of utmost importance to allow for better control of diabetes. Based on the old classification system developed by an Expert Committee in 1980 two main types of DM were proposed; 1) Insulin Dependent Diabetes Mellitus (IDDM) known as Type 1 DM (Figure 1.1) and 2) Non-Insulin Dependent Diabetes mellitus (NIDDM) known as Type 2 DM (Figure1.2) **(DEFRONZO et al., 2015; WHO, 2016)**. However, in the 1985 Study Group Report, the categorisation of Type 1 and Type 2 were excluded and only categories of IDDM and NIDDM were maintained. Moreover, a new class, known as 'Malnutrition-related Diabetes mellitus' (MRDM) was introduced, however it is no longer part of the WHO classification guide **(BILAL et al., 2018; DEFRONZO et al., 2015; WHO, 2016)**. This classification system was, however, considered misleading as the diagnosis was categorised on the basis of

the patients' need for insulin and age instead of pathological processes. Considering the limiting factors of this system the World Health Organisation proposed a new classification system from which active etiological factors and not pharmacological therapies are considered prior to diagnosis **(O'RAHILLY, 1998; WHO, 2016)**. Furthermore, from the 1980 and 1985 reports, numerous categories of diabetes comprised of other types such as Gestational DM (GDM) and Monogenic diabetes syndromes (MODY), a type of NIDDM, occurring as an effect of autosomal-dominant inheritance that cause insufficient insulin secretion **(BASTAKI, 2005; JAIN, 2012; ADA, 2018)** were recorded. At present Type 1 and Type 2 are terms commonly used to respectively depict IDDM and NIDDM **(BASTAKI, 2005)**.

1.3.1.1 Type 1 Diabetes mellitus (T1DM)

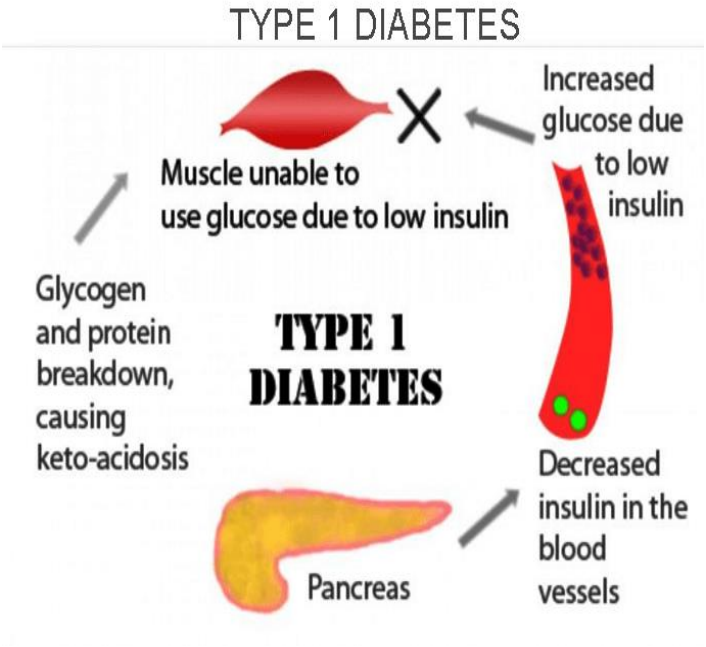

Figure 1. 1: Illustration of the pathogenesis of Type 1 Diabetes mellitus. Image retrieved from: http://digitalprinthouses.com/type-1-diabetes-type-1-diabetes/

Occasionally referred to as Juvenile diabetes, T1DM is an autoimmune disease of the β-cells responsible for the production of insulin in the body and can develop at any age **(BASTAKI, 2005; GARG, 2011; DEFRONZO et al., 2015)**. The destruction of the β-cells causes absolute

deficiency in insulin. This is known as 'idiopathic' of which the cause and pathologic processes are not known. This form is mostly common in African and Asian aboriginals **(KERNER and BRÜCKEL, 2014; DEFRONZO et al., 2015)**.

The forms of T1DM are clinically known as Type 1a, which occurs due to insufficient insulin due to the impairment of the pancreatic β-cells and affects 90% of Type 1 patients and Type 1b is considered idiopathic and affects 10% of Type 1 diabetes patients, respectively **(BASTAKI, 2005; ACHENBACH and ZIEGLER, 2005; WASSERFALL and ATKINSON, 2006)**. Currently, the exact cause of T1DM is still not clear, however, it has been associated with a number of factors such as genetics and environmental factors **(THEBERGE, 2010)**. T1DM contributes to approximately 10% of diabetes cases **(BILAL et al., 2018; GARG, 2011)**. Some of the symptoms of T1DM may occur occasionally and include dehydration (polydipsia), frequent urination (polyuria), bulimia, weight loss, change in vision and fatigue **(DEFRONZO et al., 2015; WHO, 2016).**

1.3.1.2 Type 2 Diabetes mellitus (T2DM)

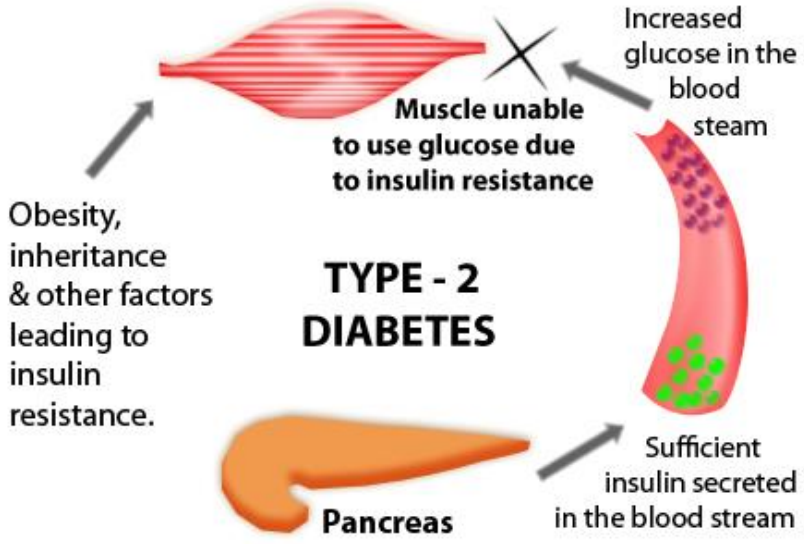

Figure 1. 2: Illustration of the pathogenesis of Type 2 Diabetes mellitus. Image retrieved from: http://www.health.am/db/more/risk-for-type-2-diabetes/

T2DM, sometimes referred to as adult-onset diabetes, is mainly observed in adults although a few incidences do occur in children **(WHO, 2016)**. T2DM stems from the body's inability to efficiently use insulin produced by β-cells. This could be a result of insulin resistance or lack of insulin sensitivity from target cell receptors **(WHO, 2016; GOBOZA et al., 2016)**. This type of diabetes occurs frequently in obese or overweight individuals, however, in this regard obesity is seen as a phenotypic expression and not necessarily a symptom of diabetes. For instance, in South Africa, studies have shown that about 87% of T2DM individuals are obese. Obesity has been regarded as an instigator for T2DM. Other factors contributing to the progression of T2DM include age and the use of antiretroviral therapy (ARVs) **(KERNER and BRÜCKEL, 2014; GOBOZA, 2016; WAUGH et al., 2007)**.

T2DM is very common and a larger number of incidents occur in urban rather than rural areas (**BASTAKI, 2005**). However, in southern Africa, the incidence of T2DM has started to increase in rural areas and this could be due to genetic defects, life style changes such as the lack of exercise, dietary changes, as well as obesity amongst individuals **(MICHAEL et al., 2000; BASTAKI, 2005; GOBOZA et al., 2016; NASRI et al., 2015)**. Typically, the symptoms of T2DM may mimic those of T1DM prior to complications related to T2DM which then arise **(WHO, 2016)**.

1.3.1.3 Gestational diabetes (GDM)

GDM occurs as a result of increased blood glucose levels during pregnancy in women without a history of prior diabetes diagnosis **(NASRI et al., 2015)**. This form of diabetes appears for a short-term period and it is diagnosed during prenatal care. Women who have gestational diabetes have about a 50% chance of acquiring T2DM in future **(BASTAKI, 2005; BELLAMY et al., 2009; WHO, 2016)**. GDM increases complications during pregnancy and delivery and these complications amongst others are associated with the observed acute foetal

birth weight **(CHINSEMBU, 2018; WHO, 2016)**. This form of diabetes occurs approximately in 4-10% of all pregnancies **(BASTAKI, 2005; NDC, 2011)**.

1.3.2 Diagnosis of DM

Based on the International Expert Committee (IEC) report of 1997, there is a set of criteria used in the diagnosis of diabetes. These include indications of increased blood glucose levels also called plasma glucose criteria. For instance:

- Fasting glucose levels are ≥126 mg/dL or a casual glucose level >11.1 mmol/L are identified **(PANINI, 2013; IEC, 2009)**.
- The 75 g oral glucose tolerance test (OGTT) which is performed through the 2 h plasma glucose (2 h PG), **(GREENBERG and SACKS, 2002, IEC, 2009)**.
- The airman first class (A1C) criteria from which a test is performed using a standardised National Glycohaemoglobin Standardization Program (NGSP) certified method; known as the Diabetes Control and Complications Trial (DCCT) assay **(ADA, 2018)**.

During pregnancy, gestational diabetes can be diagnosed using the following criteria; the "One-step" approach of 75 g OGTT or the "Two-step" approach with a 50 g (non-fasting) screening followed by a further 100 g OGTT screening for those who screened positive **(ADA, 2018)**. Due to a number of complications associated with DM the National Diabetes Data Group (NDDG) proposed criteria for diagnosing DM **(ADA, 2009)**, based on:

- Epidemiological data, related to blood glucose levels
- Detected microvascular complications

1.3.3 Symptoms and complications associated with DM

The symptoms for T1DM and T2DM are not too different but the intensity at which they present may vary **(BASTAKI, 2005)**. In T1DM, symptoms may occur promptly and are more pronounced and this, amongst others, include loss of weight, candidiasis, polyuria (excessive urination), polyphagia (intense thirstiness), polydipsia (excessive hunger) and blurred vision **(COOKE and PLOTNICK, 2008)**.

Most complications develop if diabetes is left untreated or if uncontrollable, can cause life threatening conditions **(WHO, 2016)**. These may occur as a result of postprandial hyperglycemia, excessive exercise or higher doses of antidiabetic medicine **(WHO, 2016)**. High blood glucose levels can initiate malaise such as Diabetic ketoacidosis (DKA) in both T1DM and T2DM and can further cause hyperosmolar non-ketonic coma in T2DM sufferers **(WALLACE, 2004; MOHAN, 2002)**. Hypoglycemia may occur as a result of extremely low blood glucose levels and this can happen to any diabetic individual and may cause seizures or unconsciousness **(WHO, 2016; WALLACE, 2004; MOHAN, 2002)**. Prolonged uncontrolled diabetes can cause more chronic complications such as diabetic retinopathy (damaged retina) and can cause blurred vision or blindness, diabetic neuropathy (damaged nerves) on the feet and can cause foot ulcers which may attract infections and lead to limb amputation (Table 1.1). Diabetic nephropathy (kidney damage) causes kidney failure and atherosclerosis which may lead to strokes and heart attacks **(WHO, 2016; WALLACE, 2004; MOHAN, 2002)**. These life threatening complications contribute significantly to health costs, mortality and morbidity **(WHO, 2016)**. Some of the GDM complications include congenital deformity, nervous system muscle anomalies, maternal disability, respiratory distress syndrome, stillbirth and in severe cases, perinatal death **(NDC, 2011; WHO, 2016)**.

Table 1. 1: Categorisation of diabetes associated complications **(WALLACE, 2004; MOHAN, 2002; NASRI et al., 2015).**

Acute complications	**Chronic complications**
Infections Diabetic ketoacidosis (DKA) Hyperglycemia, hyperosmolar Non ketonic coma Polydipsia, polyuria, fatigue, blurred vision	Blindness, retinopathy Neuropathy Atherosclerosis Peripheral vascular disorder, limb amputation, infection Cerebrovascular disease
Macrovascular complications	**Microvascular complications**
Stroke Heart disease, hypertension Peripheral vascular disorder Foot problems	Retinopathy and cataracts Renal disease Neuropathy Foot problems

1.3.4 Prevalence and incidence of DM

NCDs such as Diabetes mellitus have caused global concern, because over 422 million people are affected worldwide with millions more projected to become affected by the disease in the coming years. For instance, the International Diabetes Federation **(IDF, 2011)**, reported that approximately 552 million people will be affected by the year 2030 and it will become the 7th leading cause of mortality. This incidence trend was observed to be higher in the low and mid-income countries as compared to high income countries **(WHO, 2016)**. Diabetes affects individuals who are predominantly between the ages of 40-59 years **(IDF, 2011)**. According to

MANAF et al., (2016), about 11% of the pre-diabetic population are diagnosed with Type 2 diabetes annually.

Diabetes was once unheard of in the African continent, however, at present Africa is also faced with the diabetes crisis and the prevalence has abruptly increased over the years **(CHINSEMBU, 2018)**. In the Sub-Saharan Africa (SSA) region, about 10.8 million people were inflicted with diabetes in 2006 but the figure is projected to increase to 18.7 million by the year 2025, exceeding the global rise of 55% **(LEVITT, 2008)**. The burden of diabetes is set to become higher in third world countries than first world countries. In South Africa (SA), DM is the second leading cause of death with increased statistics of a 5.4% mortality rate with over two million people diagnosed as diabetic **(ERASTO et al., 2005; STATS SA, 2017)**. Higher prevalence's have been observed in the Indian and Black communities **(JOUBERT et al., 2007; SEMENYA et al., 2012)**. Provinces such as Limpopo and KwaZulu-Natal have the highest burden of DM in SA **(JOUBERT et al., 2007; SEMENYA et al., 2012)**.

1.3.5 Control and management of DM

Individuals with DM should have easy access to advanced care provided by well-trained health care providers. Although diabetes is a chronic disease, health facilities should be established to accommodate patients of DM and should be organised in order to provide interventions such as physical activity or blood glucose control treatments and allow for frequent check-ups **(WHO, 2016)**. For better management of diabetes, individuals require early diagnosis as prolonged and untreated DM can cause severe health complications **(WHO, 2016)**.

Symptoms are more pronounced in T1DM so patients are able to immediately get medical check-ups. These symptoms may include thirst, frequent urination and unexplained weight loss. However, due to the gradual progression of T2DM, symptoms may not be as pronounced until an individual start experiencing some associated complications such as blurred vision,

stroke or limb gangrene. GDM requires intense medical supervision during pregnancy. Changes in diet and often in extreme cases insulin is used to control GDM **(NDC, 2011; WHO, 2016)**.

Patient awareness about the importance of diabetes management contributes greatly in controlling diabetes. This along with changes in life style and diet can prevent the development of diabetes associated complications. Therefore, it is imperative that patients participate by taking treatments to keep their HbA1c level at 6.5% with a combination of other recommendations i.e. (exercising and eating healthy) **(GOBOZA et al., 2016; NASRI et al., 2015)**. For example, as sugar-sweet beverages (SSB) may contribute to obesity and weight gain especially in T2DM, SA has come up with a model study that projects that a 20% tax on SSB could help reduce obesity in adults thereby reducing the chances of T2DM in adults **(MANYEMA et al., 2014; HU and MALIK, 2010)**. Moreover, infections, smoking and environmental stress are some of the factors that aggravate diabetes, so families of diabetic patients are encouraged to provide support to avoid these factors on a daily basis **(GOBOZA et al., 2016; NASRI et al., 2015)**.

1.3.6 Current drugs used to treat diabetes

A number of drugs (Table 1.2 and Figure 1.4), are used in the treatment and control of diabetes and these are categorised as sulfonylureas, metformin, thiazolidinediones, α-glucosidase inhibitors and insulin **(EZURUIKE and PRIETO, 2014; STRANKS and MEYER, 2016)**.

1.3.6.1 Sulfonylureas

Sulfonylureas are a group of potent synthetic, oral anti-hyperglycemic agents available for the treatment of T2DM **(KATZUNG, 1995)**. These hypoglycemic drugs promote the production of insulin from the pancreatic β-cells by stimulating insulin sensitivity and decreasing the

secretion of hepatic glucose **(GABOZA et al., 2016; NASRI et al, 2015; KATZUNG, 1995)**. Sulfonylurea drugs have different modes of action and these include increased insulin release, decrease in serum glucagon levels and increased target cell sensitivity to insulin **(CLARK et al., 1997)**. Sulfonylurea drugs target the adenosine triphosphate potassium (ATP) sensitive channels and bind to the sulfonylurea receptors on the pancreatic β-cell plasma membrane. The targeted channel then becomes restricted and successively causes changes within the cells of the membrane triggering insulin secretion **(MODAK et al., 2007)**. Sulfonylurea drugs have, however, been associated with the onset of cardiovascular diseases **(NATHAN, 2014)**.

1.3.6.2 Metformin

Metformin (Met) belongs to the Biguanide family and is derived from the plant *Galega officinalis*. Met is a hypoglycemic drug which is currently used in the treatment of T1DM **(KLIP and LEITER, 1990; GOBOZA et al., 2016)**. Hepatic glucose production is controlled by Met by reducing the rate of the hepatic gluconeogenesis **(KRENTZ and BAILEY, 2005)**. As the first line of treatment, the drug can also be used to treat pre-diabetic patients and does not instigate weight gain but can cause side effects such as diarrhoea, abdominal pain and nausea **(MATTHAEI et al., 2000; NASRI et al., 2015)**.

1.3.6.3 Thiazolidinediones

Thiazolidinediones (TZDs) are a class of oral antidiabetic drugs which have unique functions **(BASTAKI, 2005).** TZDs are also referred to as glitazones, and their main function is to improve peripheral sensitivity of insulin in muscles, liver and fat tissues by stimulating the peroxisome proliferator-activated receptor gamma (γ) modulators **(NATHAN, 2014)**. Some of the side effects caused by these agents include extended hypoglycemia oedema and weight gain **(PHILIS-TSIMIKAS, 2013; BASTAKI, 2005)**.

1.3.6.4 α-Glucosidase inhibitors

α-Glucosidase inhibitors slow down the absorption of complex carbohydrates from the digestive tract and decrease postprandial hyperglycemia, therefore resulting in the production of a low glycaemic index **(GOBOZA et al., 2016)**. These drugs can be administered either as a monotherapy or in combination with other drugs, whether in T1DM or T2DM treatment, because they effectively reduce postprandial hyperglycemia. However, hypoglycaemia may be caused when used in combination with insulin or sulfonylurea agents **(BASTAKI, 2005; KRENTZ and BAILEY, 2005; GOBOZA et al., 2016)**. The side effects include production of excessive gas and abdominal pain **(BASTAKI, 2005; GOBOZA et al., 2016)**.

1.3.6.5 Insulin

Insulin (Figure 1.3), is a hormone produced by the pancreatic β-cells **(MEHANNA, 2005)**. The entry of glucose into the muscles, liver and fat is enabled by insulin, through the stimulation of numerous enzyme reactions from the insulin receptor **(ORCI, 1986)**. Insulin is a strong glucose reducing agent with only hypoglycemia as a dose-limiting factor, compared to the other oral hypoglycemic agents. However, some patients develop poor glycemic control **(BASTAKI, 2005)**.

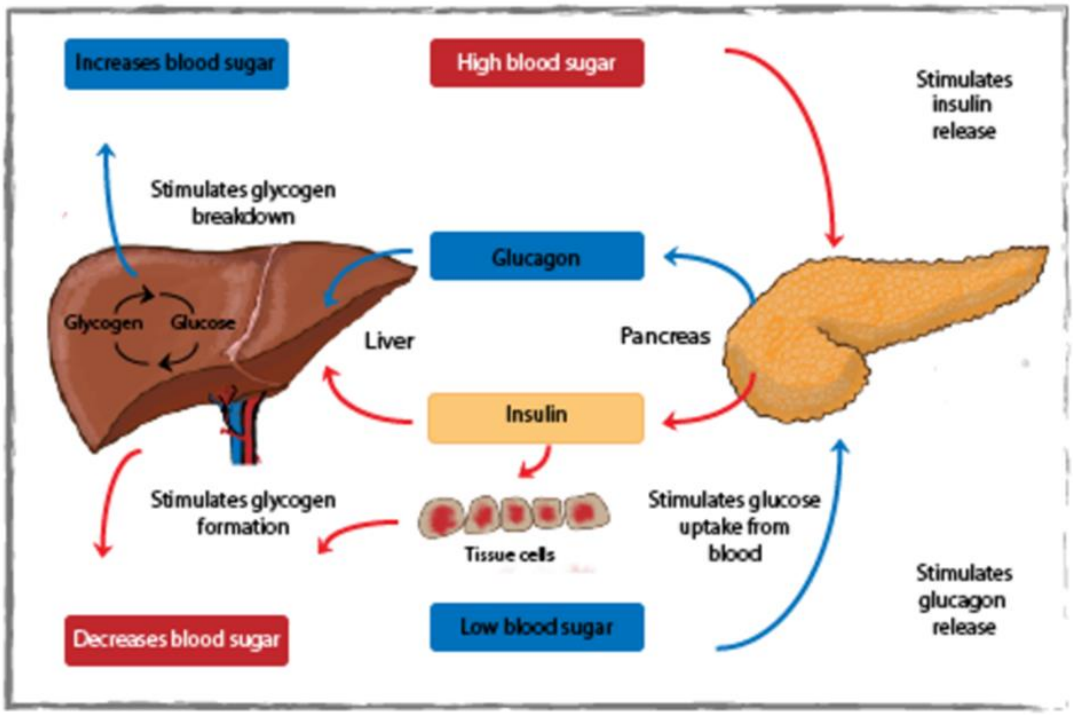

Figure 1. 3: Blood glucose regulation by insulin to halt or prevent hypoglycemia or hyperglycemia. Image retrieved from: **https://www.futurelearn.com/courses/understanding-insulin/0/steps/22457**

Patients, especially those with T2DM, may develop insulin resistance due to obesity, however, insulin resistance is seen as a sign of undesirable changes of the metabolism i.e. (CVDs, blood pressure and hyperlipidaemia). Furthermore, the development of insulin resistance in T1DM patients has been associated with immunological or non-immunological factors. Reduced insulin receptors of target cells result in resistance to the hormone. Common side effects of insulin include weight gain and hypoglycemia (**BASTAKI, 2005; MEHANNA, 2005)**.

There are several insulin treatment preparations as presented in Table 1.2; these are known as 'short-acting' preparations in which insulin absorption is slower and also takes time to reach normal prandial effects. Intermediate-acting preparations dissolve gradually and act over a longer period. Long-acting preparations are not consistent and are required between meals in very low concentrations **(KARASU et al., 1995)**.

Due to the severe side effects of the above-mentioned diabetic drugs, most patients seek alternative therapy and therefore prefer the use of medicinal plants for treatment.

Table 1. 2: Insulin analogs grouped according to duration of action **(BASTAKI, 2005).**

Short-acting insulin (2-5 h)	**Intermediate-acting insulin** (18-24 h)	**Long-acting insulin** (18-36 h)
Lispro (Humalog) Aspart (Novolog) Glulisine (Apidra) Hexamer Dry powder; Liquid suspension	Neutral Protomaine Hadegon [NPH] (Isophane) Lente	Ultralente Protoamine zinc Glargine

Figure 1. 4: Oral hypoglycemic drugs used to treat T2DM

1.4 The use of medicinal plants to treat ailments

Plants have always been used, not only for shelter and as a food supply but also as medicines to treat, prevent and cure a wide range of ailments **(SOFORWA, 1984; YAKUBU et al., 2014)**. Globally, millions of people depend on medicinal plants for health care **(CORDELL, 1995)**. Despite the multitude of antidiabetic drugs available on the market, many patients still rely on traditional medicine for their primary health care, especially in developing countries **(WHO, 2016)**.

In South Africa, traditional medicines have been a mainstay for centuries and form an essential part of various cultures **(VAN STADEN, 2008; MANDER, 1998)**. Approximately 3,000 indigenous medicinal plant species have been identified as they are habitually used, however, only a few have been validated and commercialised **(VAN WYK, 2008)**. The use of traditional medicine is still a common practice in SA and approximately 27 million people are dependent on it. The majority of South African black communities reportedly consult healers and use traditional medicine as their primary source of health care **(VAN WYK, 2008)**. For example, in KwaZulu-Natal 83% of the population is dependent on traditional medicine. Traditional healers and herbalists use various medicinal plants to heal, treat or cure numerous ailments **(MANDER, 1998; STATS SA, 2017)**.

South Africa has experienced a gradual increase in the number of traditional healers with an estimated 200, 000 actively practicing healers **(MANDER, 1998)**. Furthermore, the practicing traditional healers use various plant materials as a source of indigenous medicinal remedies **(MANDER, 1998; VAN VUUREN and VILJOEN, 2011**). The use of these indigenous medicines stems from different cultural and religious beliefs and is not only limited to the rural communities, they are also used for healing purposes in certain parts of urban communities **(DOLD and COCKS, 2002; MANDER et al., 2007; MAKUNGA et al., 2008; NDHLALA**

et al., 2011). The wide use of traditional medicine is due to the fact that they are cost effective, readily available and easily accessible and are believed to cause minimal side effects **(WHO, 2008)**.

The demand for traditional medicine is very high owing to the ever increasing human population and prevalent ailments such as diabetes **(MANDER, 1998)**. For instance, Zulu traditional healers and herbalists utilise plant material in enormous quantities to satisfy the needs of their patients, and as a result, the KwaZulu-Natal 'muti' markets reportedly trade about 400 plant species amounting to an estimated 4,300 tonnes of plant material per year **(MANDER, 1998; CUNNINGHAM, 1988)**. The demand is further propelled by the high cost of oral antidiabetic synthetic drugs, insulin and the detrimental side effects of the treatments **(BALAJI et al., 2015)**.

1.5 Drug discovery from medicinal plants

A drug according to **RATES (2001)** can be defined as "a pharmacologically active compound, which is a component of a medicine, irrespective of its natural, biotechnological or synthetic origin". Various drugs are used to prevent, treat, heal or even cure diseases. Many drugs are derived from natural sources such as plants, minerals and animals **(RATES, 2001)**. Compounds from medicinal plants are major scaffolds for the development of modern synthetic medicine and contribute largely to the pharmaceutical industries, accounting for approximately 50% of derived drugs **(PAN et al., 2013)**. Ethnopharmacology still continues the drive for the discovery of new and potent plant derived medicinal compounds **(FABRICANT and FARNSWORTH, 2001)**.

The discovery of isolated medicinal compounds from plants began in 1805 with the German pharmacist Friedrich Serürner who isolated morphine from the plant *Papaver somnifererum* **(KINGHORN, 2001)**. Isolated bioactive compounds could either be used as direct novel drugs or may serve as scaffolds for succeeding generations of drugs **(FABRICANT and FARNSWORTH, 2001)**.

Although it is of importance to devise improved natural medicines, there are numerous challenges that are encountered during the process of drug discovery which include high costs (about 800 million dollars), lengthy time periods (approximately 10 years) and insufficient yield of the natural compounds for further optimization and clinical trials **(DICKSON and GAGNON, 2004; BALUNAS and KINGHORN, 2005)**. Therefore, in order to become successful, scientists need to develop enhanced and rapid procedures to allow the drug development phase to keep up to speed with other drug discovery ventures **(BUTTLER, 2004)**.

1.6 Rationale/Problem Statement

The current drugs administered for the treatment of DM do not cure but alleviate the symptoms **(PELTZER et al., 2001)**. Thus, due to the complications and side effects associated with DM and treatment regimes, in addition to factors such as limited resources and accessibility to health facilities, medicinal plants have become an immediate source of relief and are used mainly because of their varied biological activities, availability, cost effectiveness and minimal side effects **(GURUB-FAKIM, 2006; EKOR, 2013; ANDRADE-CETTO and WIEDENFELD, 2004)**. For example, KwaZulu-Natal province has the highest unemployment rate resulting in increased poverty and consequently people do not have easy access to clinics or hospitals. The agonising waiting lines further aggravate the problem and this in turn impacts negatively on the management and control of DM because people shun visiting hospitals and

clinics **(SEMENYA et al., 2012; PILLAY et al., 2016)**. Medicinal plants are the nearest source of relief with minimal side effects. A number of these plants have shown the potential to act as blood glucose level reductants or may act as inhibitors of glucose absorption from the gastrointestinal tract thus slowing down postprandial hyperglycemia **(GURUB-FAKIM, 2006)**. It has been reported in ethnobotanical literature that approximately 800 plant species have anti-diabetic properties, though a limited number of herbs displayed antidiabetic activity when tested using current methods including screening of α-glucosidase and α-amylase enzyme inhibition **(OLARCON-AGUILERA, 2000; GURUB-FAKIM, 2006)**. Antidiabetic activities in plants have been associated with the production of chemical compounds including alkaloids, glycosides, polysaccharide, peptidoglycans, steroids, carbohydrates, amino acids and inorganic ions **(GURUB-FAKIM, 2006)**.

A large number of plants are yet to be scientifically studied and lack proper documentation **(MANDER, 1998)**. Hence there is a need to search for locally important and traditional medicinal plants used to treat diabetes. Therefore, this research aspires to identify plants which may be used successfully to treat diabetes through scientific investigation and the elucidation of active compounds to provide preliminary biological activities of antidiabetic plants and to contribute to the growing ethnopharmacology database in SA.

1.7 Plants studied

1.7.1 Morphological description and distribution of selected medicinal plants.

Bulbine natalensis also known as *Bulbine latifolia* (Asphodelaceae), is commonly known as broad-leaved bulbine in English, ibhucu in IsiZulu, rooiwortel in Afrikaans and ingcelwane in IsiXhosa. The plant is widely distributed in the eastern and northern parts of South Africa **(VAN WYK et al., 1997)**. *Bulbine natalensis* is a fast growing succulent plant with a resemblance of the aloe plant and forms solitary rosettes of up to 20 cm high. It has triangular lanceolate evergreen leaves and a floral inflorescence that contain 1-4 densely arranged flower racemes with yellow petals (Figure 1.5). The plant has a small rounded capsule fruit and fleshy, yellow roots **(VAN WYK et al., 1997)**.

Figure 1. 5: *Bulbine natalensis* Baker

Kigelia africana (Bignoniaceae) is commonly referred to as cucumber or sausage tree in English, worsboom in Afrikaans and umVungata or umFogothi in IsiZulu. The tree grows up to 20 m tall or more with a grey and smooth bark as thick as 6 mm **(ROODT, 1992; VAN WYK et al., 1997)**. This tree is distributed from KwaZulu-Natal to Tanzania and can be found along open woodland, floodplains and streams **(JOFFE, 2003)**. The tree can be both evergreen and deciduous depending on environmental seasons. The leaves are opposite or in whorls of three with pinnate oval leaflets, however, the terminal leaflet may be present or absent. The tree produces reddish bell shaped bisexual flowers with horizontal orientation growth. The fruit is a long woody berry, which hangs from the tree branches and can weigh up to 10 kg (Figure 1.6). **(JOFFE, 2003; VAN WYK et al., 1997)**.

Figure 1. 6: *Kigelia africana* (Lam.) Benth. Syn. *Kigelia pinnata* (Jacq.). Image retrieved from: https://en.wikipedia.org/wiki/Kigelia#/media/File:Kigelia_africana_compose.jpg

Leonotis leonurus (Lamiaceae) is commonly known as wild dagga or lion's ears in English; wilde dagga in Afrikaans and umcwili or umnyane in IsiZulu. It is mainly found in the grassland areas of Eastern Cape, Western Cape, Limpopo, KwaZulu-Natal and Mpumalanga **(VAN WYK et al., 2000)**. A soft-woody shrub grows up to 2 m tall. The shrub is composed of branched stems with numerous ball-shaped inflorescences of bright orange flowers. The stem of the shrub is velvety and delicate and has leaves that are bright yellow green, narrowly lanceolate with toothed margins and have a unique strong aromatic smell when crushed. Flowering time is from November to January in southern Africa (Figure 1.7) **(OYEDEMI and AFOLAYAN, 2011; http://pza.sanbi.org/leonotis-leonurus).**

Figure 1. 7: *Leonotis leonurus* (L.) R. Br.

Image retrieved from: https://www.amazon.com/Diamond-Perennials-Leonotis-Leonurus-Potted/dp/B01HST0NY8

Sclerocarya birrea (Anacardiaceae) is commonly known as marula in English, moroela in Afrikaans and umgamu in IsiZulu **(VAN WYK et al., 1997)**. The marula tree (Figure 1.8) is a

medium sized, single-stemmed, terrestrial, erect, perennial deciduous tree of about 10-15 m in height **(VAN WYK et al., 1997)**. The stem bark of the tree is flaky with a grey and pale pattern. The leaves are compound with different pairs of ovate leaflets and one terminal leaflet present. The flowers are presented as small oblong clusters with red and yellow sepals and petals respectively **(OJEWOLE, 2003)**. The fruits are pale yellow and contain a kernel with 2-4 seeds **(VAN WYK et al., 2000; OJEWOLE, 2003)**. *S. birrea* is widely distributed in Africa, from the north of Ethiopia to the south of KwaZulu-Natal (SA); west of Gambia across Central Africa to the east of Kenya and Sudan **(VAN WYK et al., 1997; BELEMTOUGRI et al., 2007)**.

Figure 1. 8: *Sclerocarya birrea* (A.Rich.) Hochst. Subsp. *caffra* (Sond.) Kokwaro. Image retrieved from: https://commons.wikimedia.org/wiki/File:Sclerocarya_birrea_habitus.jpg

1.8 Research objectives

- To investigate and validate selected medicinal plants used in South Africa to treat diabetes using *in-vitro* antidiabetic assays.
- To evaluate the antioxidant and phytochemical properties of selected plants.
- To determine whether the plant material exerts any cytotoxicity activity.
- Due to some antidiabetic complications, the antimicrobial properties of the plant species were also assessed.

CHAPTER TWO: ANTIOXIDANT ACTIVITY AND PHYTOCHEMISTRY OF SELECTED PLANT SPECIES

2.1 Introduction

Oxidative stress, an imbalance between the production of antioxidants and reactive oxygen species (ROS), in favour of the latter, results in a build-up of free radicals further complicating the health of diabetic patients **(SHORI, 2015; ASMAT et al., 2016)**. Numerous studies have shown that oxidative stress may be induced by free radicals which are produced in the event of an increase in blood glucose levels, which in turn aggravates the progression of diabetes mellitus (DM) **(JOHANSEN et al., 2005)**. Furthermore, oxidative stress has been reported to be a major contributor to the development of vascular complications, especially in Type 2 DM (T2DM) **(ASMAT et al., 2016)**. Free radicals are unstable chemical compounds and are the result of an imbalance between the production and destruction of reactive oxygen species (ROS) **(KIBITI and AFOLAYAN, 2015; OYEDEMI and AFOLAYAN, 2011)**. The presence of unpaired electrons makes ROS highly reactive molecules; these include molecules such as superoxide anions, hydroxyl nitric oxide and hydrogen peroxide radicals **(AMAROWICZ et al., 2004; OYEDEMI and AFOLAYAN, 2011)**. Reactive oxygen species, produced by the body as natural by-products, are considered a threat due to their abilities to attack and damage DNA molecules, proteins and lipids **(AMAROWICZ et al., 2004)**. Cellular oxidative damage such as these could lead to the development of several diseases such as DM, asthma, inflammation and cancer **(SKARBEZ et al., 2010)**.

The human body has a range of defence mechanisms which assist in preventing the formation of free radicals thereby alleviating oxidation damage **(AMAROWICZ et al., 2004; POLJSAK et al., 2013)**. Defence mechanisms include enzymatic and non-enzymatic antioxidants such as ascorbic acid (ASC), catalase (CAT), tocopherol, superoxide dismutase

and glutathione reductase. The disturbance of these defence functions, due to pathological processes, may lead to cellular damage **(POLJSAK et al., 2013; SHRUTHI et al., 2012)**.

The management of ROS in preventing oxidative stress is a valuable approach which may aid in managing DM and associated complications **(JOHANSEN et al., 2005)**. This may be achieved through the use of synthetic antioxidants such as butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) or the use of natural antioxidants of plant origin **(OYEDEMI and AFOLAYAN, 2011; POLJSAK et al., 2013)**. The use of natural antioxidants has become a popular choice for many people because dietary synthetic antioxidant drugs cause undesirable side effects such as heart burn; diarrhoea and may become carcinogenic or cause genotoxicity, if administered at high concentrations **(POLJSAK et al., 2013; NDHLALA et al., 2010)**. Furthermore, due to their synergistic actions, natural antioxidants from plant sources may be effective ROS scavengers **(NDHLALA et al., 2010)**.

Medicinal plants are a vital source of antioxidants. A number of studies have demonstrated that a diet rich in vegetables and fruits as well as consumption of medicinal plants with high antioxidant content may improve hyperglycemia and slow down the development of complications associated with diabetes **(NISHIKAWA and ARAKI, 2013; NASRI et al., 2015)**. The strong medicinal and antioxidant activities of plants may be attributed to the presence of secondary metabolites such as phenolics or even nitrogen containing compounds as well as carotenoids **(ERDEMOGLU et al., 2006; SAINI et al., 2013)**. For example, phenolic compounds have been reported to demonstrate inhibitory activity against carbohydrate related enzymes i.e. (α-amylase and α-glucosidase) and may contribute to the treatment of increased blood glucose levels after a meal or the management of DM with minimal side effects **(McCUE and SHETTY, 2004; RANILLA et al., 2010)**. Therefore, the aim of this study was to investigate the phytochemical compounds and antioxidant activities of

the selected plant species, which are used traditionally to combat DM and associated complications.

2.2 Materials and Methods

2.2.1 Plant selection and collection

Four plants with reported antidiabetic activity were selected based on the available indigenous ethnopharmacological literature of medicinal plants used by various tribes in South Africa for the treatment of diabetes and related symptoms **(HUTCHINGS et al., 1996; VAN WYK et al., 1997)**. Material of the plant species were collected between February and April 2018. Voucher specimens were deposited at the Herbarium [Natal university (NU)] of the University of KwaZulu-Natal, Pietermaritzburg for botanical verification and future reference (Table 2.1).

Table 2. 1: Plant species investigated in this study

Plant species	Parts used	Place of collection	Voucher number	Traditional use	References
Bulbine natalensis Baker.	Leaves and Roots	UKZN Botanical garden	MFRatsoma 01 NU	The leaves and roots are used to treat eczema, syphilis, vomiting, diabetes and diarrhoea	**WATT and BREYER-BRANDWIJK, 1962; OYEDEMI et al., 2009.**
Kigelia africana (Lam.) Benth.	Leaves and Fruit	UKZN Botanical garden	MFRatsoma 02 NU	Powdered fruit and leaves are used to treat ulcers, syphilis, diabetes, rheumatism, acne and as a purgative.	**HUTCHINGS et al., 1996.**
Leonotis leonurus (L.) R. Br.	Leaves	Mkhabathini farm KZN	AYoung 2999	The leaf concoctions are used to relieve headaches, cough, cold, influenza, diabetes, delayed menstruation, constipation and chest infections	**BRYANT, 1966; VAN WYK et al., 2000; HUTCHINGS et al., 1996.**
Sclerocarya birrea (A.Rich.) Hochst subsp caffra (Sond) Kokwaro.	Stem bark	Ga-Mothapo village	MFRatsoma 03 NU	Stem bark decoction is used to treat diarrhoea, gangerous rectis, malaria and diabetes	**ELOFF, 2001; BRYANT, 1966; HUTCHINGS et al., 1996; DIMO et al., 2007.**

2.2.2 Extract preparation

The plants were separated into different parts (leaves, roots, fruit and stem bark). The different plant organs were thoroughly washed under running tap water to remove contaminants and dried over a period of five days at 50 °C, in an oven. The dried plant material was ground into fine powders using a grinder (IKA®, USA) and stored in brown paper bags in cool temperatures.

Phenolic compounds were extracted from respective ground plant material as described by **MAKKAR (2000)**. The ground plant material (1 g/ 20 mL) were extracted with 50% methanol in a cold sonication bath (Branson model 5210, Branson ultrasonic B.V Soest, Netherlands) for 20 min. The resultant extract was filtered under vacuum through Whatman No.1 filter paper and the respective filtrates were used immediately for preliminary screening of phytochemical compounds and quantifications of polyphenolic content (i.e. total phenolic content; condensed tannins and flavonoid content).

Extracts used to test for biological activities were extracted in the following manner; different solvents exhibiting a range of different polarities were used, namely petroleum ether (PE); dichloromethane (DCM); acetone (ACE) and water. The ground plant material was extracted with the solvents, at different ratios as per the following; PE (20 g / 200 mL), DCM (10 g/ 100 mL), ACE (5 g/ 50 mL) and water (5 g/ 50 mL). The mixtures except for water, were placed on a rotary shaker (Edmund Bühler, Tübingen, German) for 24 h and the resultant suspension was sonicated for 1 h in a cold water sonication bath. The maceration was then filtered using Whatman No.1 filter paper and concentrated under vacuum using a Rotary evaporator. The concentrates were then dried at room temperature under a stream of cold air and later kept at 10 °C, in the dark to prevent degradation of light sensitive compounds. Water extracts were

collected in pre-weighted glass jars that were stored at -20 °C and once they were frozen the extracts were lyophilised using a Virtis Bench Top freeze dryer. The different dried extracts were resuspended to known concentrations prior to analysis of biological activity.

2.2.3 Antioxidant activity assays

2.2.3.1 β-Carotene/linoleic acid (BCA) model system

The β-Carotene/linoleic acid oxidation suspension or inhibition was determined according to the method described by **AMAROWICZ et al. (2004)**. Sample extracts and BHT (positive control) were dissolved in 50% methanol to a known concentration of 7 mg/mL. In a brown Schott bottle, 10 mg of β-Carotene was dissolved in 10 mL chloroform. The excess chloroform was evaporated under vacuum, resulting in the formation of a thin film of β-Carotene. Immediately Linoleic acid (200 µL) and Tween 20 (2 mL) were then added to the thin film of β-Carotene and mixed with aerated distilled water (497.8 mL), yielding a final β-Carotene concentration of 20 µg/mL. An orange-coloured emulsion was formed from the saturated oxygen by vigorously agitating the mixture. Soon after preparation, 4.8 mL of the emulsion was dispensed in a test tube, followed by the addition of either sample plant extracts or BHT (200 µL, 7 mg/mL), giving a final concentration of 280 µg/mL in the reaction mixture. The absorbance for each reaction was measured immediately (t = 0) at 470 nm [Cary 50 UV-visible spectrophotometer (Varian, Australia)] following which, the mixture was incubated in a water bath at 50 °C. The absorbance of each reaction mixture was measured every 30 min over a period of 180 min. Tween 20 was used to blank the spectrophotometer. The negative control was prepared by replacing sample extract with 50% methanol. The rate of bleaching was calculated using the following equation:

$$\text{Rate of β-Carotene bleaching} = [\ln (A_{t=0}/A_{t=t})] \text{ X } 1/t$$

Where $A_{t=0}$ is the absorbance of the emulsion at 0 min; $A_{t=t}$ is the absorbance at time *t* (30, 60, 90 min). The calculated average rate of β-Carotene bleaching was based on rates at 30, 60 and 90 min. The obtained calculated average rates were then used to determine the antioxidant activity (ANT) of sample extracts and are expressed as percentage inhibition of the rate of β-Carotene bleaching. The following formula was used:

$$\%ANT = (R_{control} - R_{sample} / R_{control}) X100$$

Where $R_{control}$ and R_{sample} represent average β-Carotene bleaching rates of negative control and plant extracts, respectively. Oxidation rate ratio (ORR) was calculated, to further express antioxidant activity of sample extracts using the formula:

$$ORR = R_{sample} / R_{control}$$

Antioxidant activity (AA) was calculated as described by **BRACA et al. (2003)** based on the inhibition of coupled oxidation of β-Carotene and linoleic acid against the negative control at *t* = 60 min and *t* = 120 min, the following formula was applied:

$$\%AA = [1- (A_0- A_t)/ (A_{00} - A_{0t})] X 100$$

Where A_0 is the initial absorbance of sample extract prior to incubation; A_t is the absorbance at time *t* = 60 and 120 min for sample extract; A_{00} and A_{0t} represent the absorbance of the negative control at the beginning of incubation and at time *t* = 60 and 120 min, respectively.

2.2.3.2 DPPH (1-1- diphenyl-1-picryhydrazyl) radical scavenging activity

The DPPH technique is reported as the easiest and most widely used method for screening antioxidant abilities in foods and plant products **(BOLIGON et al., 2014)**. The free radical scavenging activity of plant extracts on the stable radical (DPPH) was estimated by the method established by **KARIOTI et al. (2004)**, with modifications. Independently, the sample extracts

or standard antioxidant were suspended in 50% methanol to known concentrations beginning with the highest concentration at 50 mg/mL. Independently, 15 µL of each of the samples were diluted with 735 µL of 50% methanol and added to 750 µL (50 µM in methanol) of a freshly prepared methanolic DPPH solution (0.1 mM; prepared in a brown Schott bottle), to give a final volume of 1.5 mL in the test tube. The reaction was performed under dim light and was incubated in the dark, at room temperature for a period of 30 min. The discolouration of the purple DPPH colour in the reaction mixture was measured at 517 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). Absolute methanol was used as the blank solution. Standard antioxidants ascorbic acid (ASC) and butylated hydroxyltoluene (BHT), were used as positive controls. A negative control was prepared by replacing the sample extract or standard antioxidant with 50% methanol. Background correction of the sample absorbance (DPPH absent) was completed by subtracting the absorbance readings of sample extract in methanol only, from corresponding readings of those obtained in the presence of DPPH. The assay was completed in triplicate. This experiment was repeated thrice. The free radical scavenging activity (RSA) was calculated according to the formula:

$$\%RSA = 100 \text{ X } (1 - A_E/A_D)$$

Where A_E is the absorbance of the sample or standard antioxidant and A_D is the absorbance of the negative control. Radical scavenging activity (%) was plotted against concentration of sample extract. The concentration required to decrease the absorbance of DPPH by 50% (IC_{50}) was calculated using Graph PadPrism 5.

2.2.3.3 Ferric cyanide (Fe^{3+}) reducing antioxidant power (FRAP) assay

The FRAP assay is a simple, cost effective and efficient spectrophotometric technique **(BOLIGON et al., 2014)**. The ferric reducing power of the sample extracts was measured according to the method determined by **LIM et al. (2009)**, with modifications. Sample extracts

or standard antioxidant (BHT) were independently dissolved in 50% methanol (50 mg/mL), from which 30 µL was added to a 96-well micro-plate in triplicate and serially diluted (two-fold) downwards on the plate. To each well, 40 µL of potassium phosphate buffer (0.2 M, pH 7.2) and 40 µL of potassium ferricyanide (1% in phosphate buffer, w/v) were sequentially added. The microplate was then covered with foil and incubated at 50 $^{\circ}$C for 20 min. Post incubation, 40 µL of trichloroacetic acid (10% in phosphate buffer, w/v), 150 µL of distilled water and finally, 30 µL ferric chloride (0.1% in phosphate buffer w/v) were added sequentially. The microplate was then incubated at room temperature for 30 min in the dark. Absorbance of the reduced Fe^{3+}/ ferricyanide complex to the ferrous (Fe^{2+}) form, was measured at 630 nm using a micro-plate reader (Opsys MR^{TM} micro-plate reader, Dynex Technologies Inc. Chantailly, VA). The potential ferric reducing power of the sample extracts and BHT were expressed graphically by plotting the absorbance against concentration.

2.2.3.4 Hydrogen peroxidase (H_2O_2) antioxidant assay

The potential scavenging activity of the different plant extracts towards hydrogen peroxide was determined according to **RUCH et al. (1989)** with modification as per **NGONDA (2013)**. The solution of hydrogen peroxide (40 mM) was prepared in potassium phosphate buffer (0.2 M, pH 7.4). In test tubes, the plant extracts or standard drug (ASC/ BHT) (50 mg/mL) dissolved in 50% methanol, were further diluted with the phosphate buffer (1 mL), to which 0.6 mL of the hydrogen peroxide (40 mM) solution was added. The final mixture was incubated for 10 min in a dark room at room temperature. The concentration of hydrogen peroxide in the solution was determined by measuring the absorbance at 560 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia) against a blank solution of the phosphate buffer. The negative control contained phosphate buffer instead of the sample extract or standard drug. The

hydrogen peroxide scavenging activity (%) of the extracts were compared to a reference drug ASC and BHT and was calculated using the following formula:

$$\%\text{Scavenged } (H_2O_2) = A_C - A_S / A_C \times 100$$

Where A_C is the absorbance of the control and A_S is the absorbance of the extract or the standard drugs. The concentration required to decrease the absorbance of H_2O_2 by 50% (IC_{50}) was calculated using Graph PadPrism 5.

2.2.4 Phytochemical analysis

Phytochemicals occur naturally in plants **(ELEAZU et al., 2012)**. Plants produce and store a wide variety of groups of chemical compounds known as secondary metabolites. The occurrence of secondary metabolites in plants varies according to the species, ecology and biochemical differentiation **(GURIB-FAKIM, 2006; LEWINSOHN and GIJZEN, 2009)**. Plant secondary metabolites have long been employed by humans as stimulants and medicines **(CROZIER et al., 2006)**. Numerous secondary metabolites including tannins, alkaloids, glycosides, volatile oils and resins have been found to be biologically active, affording various medicinal uses **(NAMDEO, 2007)**. Secondary metabolites are grouped into three main classes, namely; phenolics, saponins and alkaloids **(CROZIER et al., 2006)**. Plant phytochemicals are screened because they are important leads for the discovery and development of novel drugs **(AZWANIDA, 2015)**.

2.2.4.1 Phytochemical screening

The preliminary phytochemical screening of crude extracts was determined by using the standard qualitative methods as described by **EDEOGA et al. (2005)** and **VERMA et al. (2013)**.

2.2.4.1.1 Test for flavonoids

One millilitre of the methanol filtrate was added into a tube and 5 drops of 1% v/v ammonium solution was added. The presence of flavonoids would be marked by a yellow discolouration of the respective filtrates.

2.2.4.1.2 Test for alkaloids

Five millilitres of each filtrate was added into 2 mL of hydrochloric acid. To this mixture 1 mL of prepared Dragendorff's reagent was added. An immediate change in colour (orange or red) would indicate the presence of alkaloids.

2.2.4.1.3 Test for terpenoids

Five millilitres of each methanolic filtrate was added to 2 mL of trichloromethane in a test tube, to which, 3 mL of concentrated sulfuric acid was added slowly, forming a separate layer. The presence of terpenoids was indicated by a reddish-brown colouration on the interface.

2.2.4.1.4 Test for tannins and phenolic compounds (Ferric chloride test)

In test tubes, 2 mL of each filtrate was added to 2 mL of water into which, 2-3 drops of 5% ferric chloride was added. A green or blue precipitate indicated the presence of tannins or phenols, respectively.

2.2.4.1.5 Test for carbohydrates (Benedict's test)

Five hundred microliters of each filtrate as well as Benedict's reagent (www.philipharris.co.uk) were added to test tubes which were brought to boil at 100 ^{o}C in a water bath for 5 min. The presence of carbohydrates was indicated by a bluish to green solution while a brown to black colouration would indicate the presence of flavonoids as well.

2.2.4.1.6 Test for steroids (Salkowski test)

One millilitre of each filtrate was added into 10 mL chloroform and similar amount of concentrated sulfuric acid was carefully added down the side of the test tube. To indicate the presence of steroids, the upper layer should turn red and the sulfuric acid layer should display a yellow to green colouration.

2.2.5 Quantification of polyphenolic compounds

2.2.5.1 Total phenolic content

The total phenolic contents of selected crude extracts were determined using the Folin-Ciocalteu (Folin-C) method as described by **MAKKAR (2000)**, with modifications. The reaction mixture contained 50 µL of the respective sample filtrates, 950 µL of distilled water, 500 µL of 1N Folin-C phenol reagent and 2.5 mL of 2% w/v sodium carbonate. This mixture was then incubated at room temperature for 40 min. Gallic acid (0.1 mg/mL) was used as a standard. Absorbance of the reaction mixture was measured at 725 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). The same procedure was applied to the standard solution of Gallic acid (GA) and a standard curve was obtained. The total phenolic content was expressed as mg/mL Gallic acid equivalents (GAE) per mg/g dry weight of the crude extracts of selected plants. GAE values were reported as X±SE of the triplicate reactions performed.

2.2.5.2 Condensed tannins

Quantification of total condensed tannins was done using the method described by **MAKKAR (2000)**, with modifications. Three millilitres of butanol-HCL reagent (95:5 v/v) were added to 500 µL of each crude extract. Subsequently, 0.1 mL ferric reagent (0.2% w/v ferric ammonium sulphate in 2N HCL) was added. The reaction mixture was vortexed and incubated in a boiling water bath for 1 h. Cyanidin chloride (CC) was used as a standard. The absorbance of the post-

incubation reaction mixture was measured at 550 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). Absorbance was measured against a blank mixture that contained the sample extract (500 µL), butanol-HCL reagent (3 mL) and ferric reagent (100 µL) without heating. The assay was performed in triplicate. The concentration of condensed tannins was expressed as Cyanidin chloride equivalent mg/mL (CCE) per mg/g dry weight of the crude extracts.

2.2.5.3 Flavonoid content

The determination of flavonoids was measured using the method described by **MAKKAR (2000)**, with modifications. The respective plant filtrates (250 µL) were independently diluted with distilled water to a volume of 1 mL in a test tube. To this mixture, 5% w/v sodium nitrate (75 µL), 75 µL of 10% w/v aluminium chloride, 1M sodium hydroxide (500 µL) and 0.6 mL of distilled water were added. Catechin (C) was used as a reference compound (0.1 mg/mL). The reaction mixture was measured immediately at 510 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). A reaction mixture containing 50% methanol without the extract was used as a blank. The same procedure was applied to the standard solution of Catechin equivalent (CE) and a standard curve was obtained. The flavonoid content was expressed as mg/mL Catechin equivalent (CE) per mg/g dry weight of the crude extracts of selected plants. All tests were carried out in triplicate. CE values were reported as X±SE of the triplicate reactions performed.

2.2.6 Statistical analysis

The obtained enzyme data of the plant extracts were subjected to a One-way analysis of variance (ANOVA) using IBM SPSS software version 21 for Windows. Significant differences ($p = 0.05$) of the means were separated using Duncan's Multiple Range Tests and IC_{50} values were conducted on GraphPad Prism Version 4.00 for Windows (GraphPad, Software Inc.)

2.4 Results and Discussion

2.4.1 β-Carotene and linoleic acid activity of plant extracts

Plant extracts were measured for their ability to delay or prevent the coupled oxidation of β-Carotene and linoleic acid and the data is presented in Table 2.2. The antioxidant activity based on the average rate of β-Carotene bleaching was high for the acetone and water extracts of roots of *B. natalensis* (86.49 and 70.89%, respectively); water extracts of leaves of *K. africana* (76.49%); water extracts of leaves of *L. leonurus* (80.16%) and acetone and water extracts of stem bark of *S. birrea* (94.58 and 84.81% , respectively) are comparable to the standard control BHT (81.75%). The PE extracts of *L. leonurus* leaves and *S. birrea* stem bark did not show any ability to reduce the bleaching of β-Carotene. The oxidation rate ratio (ORR), associated with the antioxidant capacity (ANT %) of the plant extracts ranged from 0.05 to 0.94. Antioxidant activity (AA) based on the inhibition of the oxidation of β-Carotene was further determined at t = 60 and 120 min. The antioxidant activity ranged from 2.71 to 76.76% (t=60 min) and 0.18 to 55.92 (t=120). However, several extracts including PE extracts of *S. birrea* stem bark (t=60 min) and *B. natalensis* roots; acetone and DCM extracts of *K. africana* leaves; DCM, ACE and water extracts of fruits from *K. africana*; PE extracts of *L. leonurus* leaves, showed low activity while the leaves of *K. africana* displayed a weak ability to limit β-Carotene bleaching.

Table 2. 2: The ability of plant extracts in delaying β-Carotene bleaching

Plant species	Part used	Solvent	% ANT	ORR	AA60	AA120
Bulbine natalensis Baker.	Leaves	PE	37.68±0.30^{j}	0.62±0.003^{f}	13.08±1.15^{lm}	2.60±0.83^{j}
		DCM	39.36±0.12^{ij}	0.61±0.001^{fg}	15.52±0.31^{k}	11.04±0.15^{g}
		ACE	28.87±3.54^{l}	0.71±0.004^{d}	7.61±0.62^{o}	8.77±4.97^{gh}
		Water	33.65±0.24^{k}	0.66±0.002^{e}	14.32±0.79^{kl}	0.18±0.02^{k}
	Roots	PE	31.75±0.17^{k}	0.68±0.002^{e}	6.48±0.38^{op}	NA
		DCM	52.52±0.09^{f}	0.47±0.001^{j}	24.16±0.29^{h}	23.14±0.248^{e}
		ACE	86.49±0.18^{b}	0.14±0.002^{n}	70.30±0.53^{b}	44.28±0.779^{bc}
		Water	70.89±0.12^{e}	0.29±0.001^{k}	59.63±0.38^{e}	43.75±0.25^{bc}
Kigelia africana (Lam.) Benth.	Leaves	PE	16.61± 0.13^{n}	0.83± 0.001^{b}	12.74±0.22^{m}	3.29±0.22^{ij}
		DCM	24.33±0.13^{m}	0.76±0.001^{c}	11.15±0.31^{n}	NA
		ACE	5.92±0.19^{o}	0.94±0.002^{a}	5.54±0.14^{pq}	NA
		Water	76.49±0.05^{d}	0.24±0.000^{l}	62.39±0.20^{d}	42.15±0.27^{c}
	Fruit	PE	24.26±0.19^{m}	0.76±0.002^{c}	20.02±0.20^{i}	7.61±0.10^{ghi}
		DCM	6.39±0.23^{o}	0.94±0.002^{a}	4.25±0.647^{q}	NA
BHT (control)			81.75±0.83^{c}	0.18±0.000^{m}	56.63±0.28^{f}	46.71±0.22^{bc}

Table 2. 2: Continued

Plant species	Part used	Solvent	% ANT	ORR	AA60	AA120
		ACE	44.90±0.28^{h}	0.55±0.003^{h}	14.21±0.66^{kl}	NA
		Water	45.50±0.18^{h}	0.55±0.002^{h}	12.07±0.17^{mn}	NA
Leonotis leonurus (L.) R.Br.	Leaves	PE	NA	NA	2.71±0.24^{r}	NA
		DCM	48.39±0.10^{g}	0.52±0.001^{i}	42.32±0.27^{g}	5.14±0.28^{hij}
		ACE	48.17±0.28^{g}	0.52±0.002^{i}	18.02±0.56^{j}	16.00±0.59^{f}
		Water	80.16±0.10^{c}	0.20±0.000^{m}	56.71±0.30^{f}	32.82±0.29^{d}
Sclerocarya birrea (A.Rich) Hoschst.	Stem bark	PE	NA	NA	NA	NA
		DCM	40.92±0.12^{i}	0.59±0.001^{g}	15.22±0.26^{k}	7.72±0.35^{ghi}
		ACE	94.58±0.16^{a}	0.05±0.002^{o}	68.55±0.56^{c}	55.92±2.13^{a}
		Water	84.81±0.23^{b}	0.15±0.002^{n}	76.76±0.22^{a}	47.15±2.15^{b}
BHT			81.75±0.83^{c}	0.18±0.000^{m}	56.63±0.28^{f}	46.71±0.22^{bc}

Values indicate mean ± SE of three replicates. PE= petroleum ether, DCM= dichloromethane, ACE= acetone, BH = butylated hydroxyltoluene. % ANT = Antioxidant activity calculated according to the rate of β-Carotene bleaching at t = 60- and 120-min. ORR: oxidation rate ratio, the lower the value the stronger the activity. AA60, AA120 = % antioxidant activity of the extract or BHT at t = 60- or 120-min. NA: extracts with no activity. Different letters in the same column indicate significant differences at the 5% level of significance.

2.4.2 DPPH activity of plant extracts

The results pertaining to the antioxidant activity of plant extracts as determined by the DPPH radical scavenging assay are illustrated in Table 2.3. The activity varied amongst the different plant organs and the solvent used to extract them. Potent radical scavenging activity was observed for water and ACE leaf and root extracts of *B. natalensis* (89.94 and 90.31%, respectively), DCM and water leaf extracts of *K. africana* (94.82 and 97.05%, respectively), ACE and water extracts of the fruit (98.04 and 100.00%, respectively) of *K. africana* DCM, ACE and water extracts of leaves of *L. leonurus* (80.74; 89.48 and 92.98%, respectively) and ACE and water extracts of *S. birrea* stem bark (99.09 and 96.83%, respectively) comparable to the standards. Furthermore, the IC_{50} values of the extracts ranged from 0.01 to 0.22 mg/mL (Table 2.3), with water extracts of *B. natalensis* leaves, DCM and water extracts of the leaves of *K. africana*, water extracts of the fruit of K. *africana* and the ACE and water extracts of *S. birrea* stem bark exhibited moderate inhibitory concentrations, while ACE extract of fruit of *K. africana* and water extracts of leaves of *L. leonurus* possessed lower IC_{50} values that are not significantly different compared to the standards, ASC and BHT (0.02 and 0.02 mg/mL, respectively). Extremely low antioxidant activity represented by percentage antioxidant activity below 50% at the highest concentration tested, were recorded as not active (NA).

2.4.3 H_2O_2 activity of plant extracts

The ability of the plant extracts to reduce H_2O_2 is represented in Table 2.3. Activities ranging from strong to weak were observed for the plant extracts. The ACE extract of roots of *B. natalensis* (35.18%), water extracted fruit from *K. africana* (21.12%) and ACE and water extracts of *S. birrea* stem bark (22.64 and 30.38%, respectively) demonstrated a strong ability to quench H_2O_2. Water extracts of all the plants displayed moderate to strong activity. However, except for the above mentioned extracts, organic extracts of the plants showed very weak

activity as compared to that of the standard drugs. The IC_{50} values ranged from 0.33 to 0.01 mg/mL.

Table 2. 3: Radical scavenging activity (%) of DPPH and H_2O_2 and their respective IC_{50} values (mg/mL**)**

			DPPH		H_2O_2	
Plant species	**Plant part**	**solvent**	**% RSA**	**IC_{50}**	**% H_2O_2**	**IC_{50}**
Bulbine natalensis	Leaves	PE	13.02±0.83^{jkl}	NA	99.61±0.023^{a}	NA
		DCM	29.82±3.69^{h}	NA	97.20±0.27^{bc}	NA
		ACE	37.03±2.95^{g}	NA	94.78±0.08^{d}	NA
		Water	89.94±0.24^{bc}	0.07±0.004^{bcde}	42.18±0.49^{l}	0.33±0.000^{b}
	Roots	PE	10.35±1.02^{kl}	NA	98.93±0.00^{ab}	NA
		DCM	20.84±1.67^{i}	NA	90.10±0.12^{d}	NA
		ACE	90.31±0.18^{bc}	0.12±0.007^{abcde}	35.87±0.09^{m}	0.26±0.026^{b}
		Water	57.76±1.69^{f}	0.11±0.013^{abcde}	60.57±0.20^{j}	0.32±0.003^{b}
Kigelia africana	Leaves	PE	52.67±2.76^{f}	0.22±0.150^{a}	64.56±0.13^{i}	0.31±0.001^{b}
		DCM	94.82±1.28^{abc}	0.05±0.004^{bcde}	74.49±0.37^{h}	NA
		ACE	18.48±0.62^{ij}	NA	79.45±0.03^{g}	NA
		Water	97.05±1.00^{ab}	0.03±0.003^{cde}	50.51±0.24^{k}	0.32±0.003^{b}
	Fruit	PE	8.22±0.34^{l}	NA	89.14±0.12^{e}	NA
		DCM	65.93±6.27^{e}	0.14±0.022^{abcd}	94.29±0.03^{d}	NA
		ACE	98.04±0.49^{a}	0.01±0.001^{de}	75.77±0.10^{h}	0.01±0.004^{d}
		Water	100.00±0.00^{a}	0.08±0.002^{bcde}	21.12±0.60^{o}	0.33±0.000^{b}
ASC			92.88±0.45 ^{abc}	0.02±0.003^{de}	43.00±2.24^{l}	0.39±0.000^{a}
BHT			100.00±0.00^{a}	0.02±0.003^{de}	62.81±0.73^{i}	0.27±0.037^{c}

Table 2.3: Continued

Plant species	Plant part	solvent	DPPH		H_2O_2	
			% RSA	IC_{50}	% H_2O_2	IC_{50}
Leonotis leonurus	Leaves	PE	17.13 ± 1.09^{ijk}	NA	97.69 ± 0.011^{abc}	NA
		DCM	80.74 ± 1.27^{d}	0.17 ± 0.006^{ab}	95.65 ± 0.014^{cd}	NA
		ACE	89.48 ± 3.09^{c}	0.16 ± 0.006^{abc}	99.15 ± 0.00^{abc}	NA
		Water	92.98 ± 2.93^{abc}	0.01 ± 0.003^{de}	50.55 ± 0.15^{k}	0.32 ± 0.000^{b}
Sclerocarya birrea	Stem bark	PE	41.99 ± 3.83^{g}	NA	94.72 ± 2.31^{d}	NA
		DCM	43.39 ± 4.26^{g}	NA	87.31 ± 1.24^{ef}	NA
		ACE	99.09 ± 0.22^{a}	0.03 ± 0.005^{de}	22.64 ± 0.07^{o}	0.13 ± 0.016^{d}
		Water	96.83 ± 0.54^{abc}	0.02 ± 0.001^{de}	30.38 ± 0.04^{n}	0.31 ± 0.000^{b}
ASC			92.88 ± 0.45^{abc}	0.02 ± 0.003^{de}	43.00 ± 2.24^{l}	0.39 ± 0.000^{a}
BHT			100.00 ± 0.00^{a}	0.02 ± 0.003^{de}	62.81 ± 0.73^{i}	0.27 ± 0.037^{c}

PE=petroleum ether, DCM= dichloromethane, ACE= acetone, ASC= ascorbic acid, BHT= butylated hydroxyltoluene. Values indicate mean ± SE of three replicates. Different letters associated with % RSA and % H_2O_2 indicate significant differences at the 5% level of significance. IC_{50}= sufficient concentration to obtain 50% of a maximum scavenging capacity; NA= Not active

2.4.4 FRAP activity of plant extracts

Antioxidant activity of the plant extracts based on their ability to reduce ferricyanide (Fe^{3+}) complexes in a solution to its ferrous form (Fe^{2+}) is presented in Figure 2.1. Strong antioxidants reduce the Fe^{3+} complex resulting in a Perl's Prussian blue colour change that was detected spectrophotometrically at 630 nm **(NDHLALA et al., 2014)**. Antioxidant activity varied significantly between extracts at the concentrations at which they were tested. Plant extracts exhibiting the highest reducing power included PE extracts of fruit of *K. africana*; DCM extracts of leaves of *L. leonurus* and ACE and water extracts of *S. birrea* stem bark. Plant extracts displaying the weakest reducing power at the highest concentrations tested (12.5 mg/mL) were PE and DCM extracts of *S. birrea* stem bark; ACE leaf extracts of *K. africana* and water extract of leaves of *B. natalensis*.

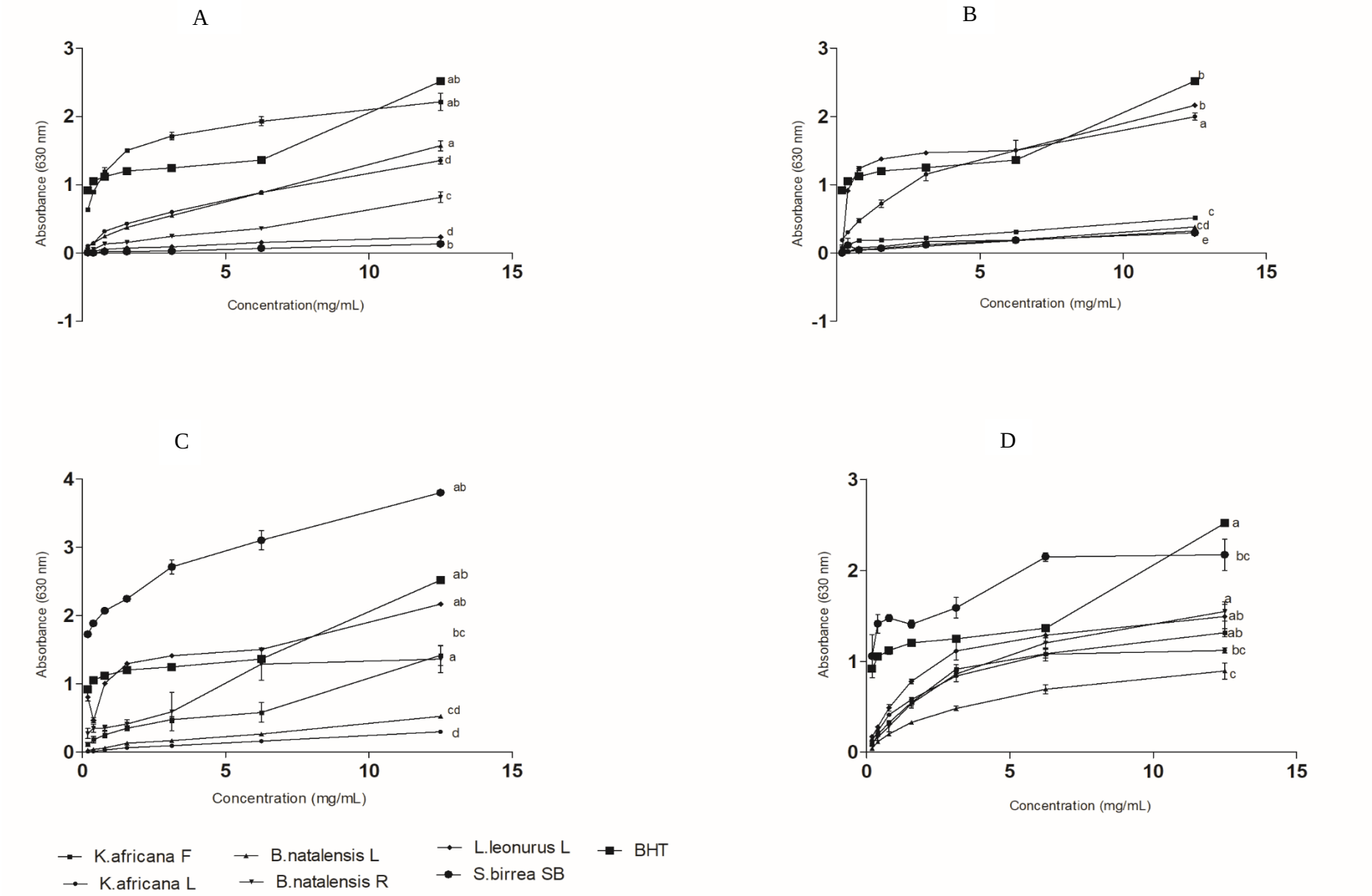

Figure 2. 1: The dose-dependent ferric reducing antioxidant power of plant extracts. BHT= butylated hydroxyltoluene. Solvents of the extracts represented above A= petroleum ether; B= dichloromethane; C= acetone, D= water. Values indicate mean ± SE of three replicates. Different letters between samples indicate significant differences at the 5% level of significance.

2.4.5 Total phenolic content of plant extracts

This method employed the use of Folin-C reagent and was established on the grounds of the oxidation reduction principle. This technique for determining total phenolic content in plants was used because it is reproducible and highly sensitive **(MAKKAR et al., 2007)**. The total phenolic contents (mg GAE/g DW) of the different plant extracts are presented in Figure 2.2. All the plant extracts exhibited different quantities of phenolic compounds, the highest of which was observed in stem bark of *S. birrea* (41,21 mg GAE/g DW), while roots of *B. natalensis*, showed the lowest total phenolic content (5,31 mg GAE/g DW).

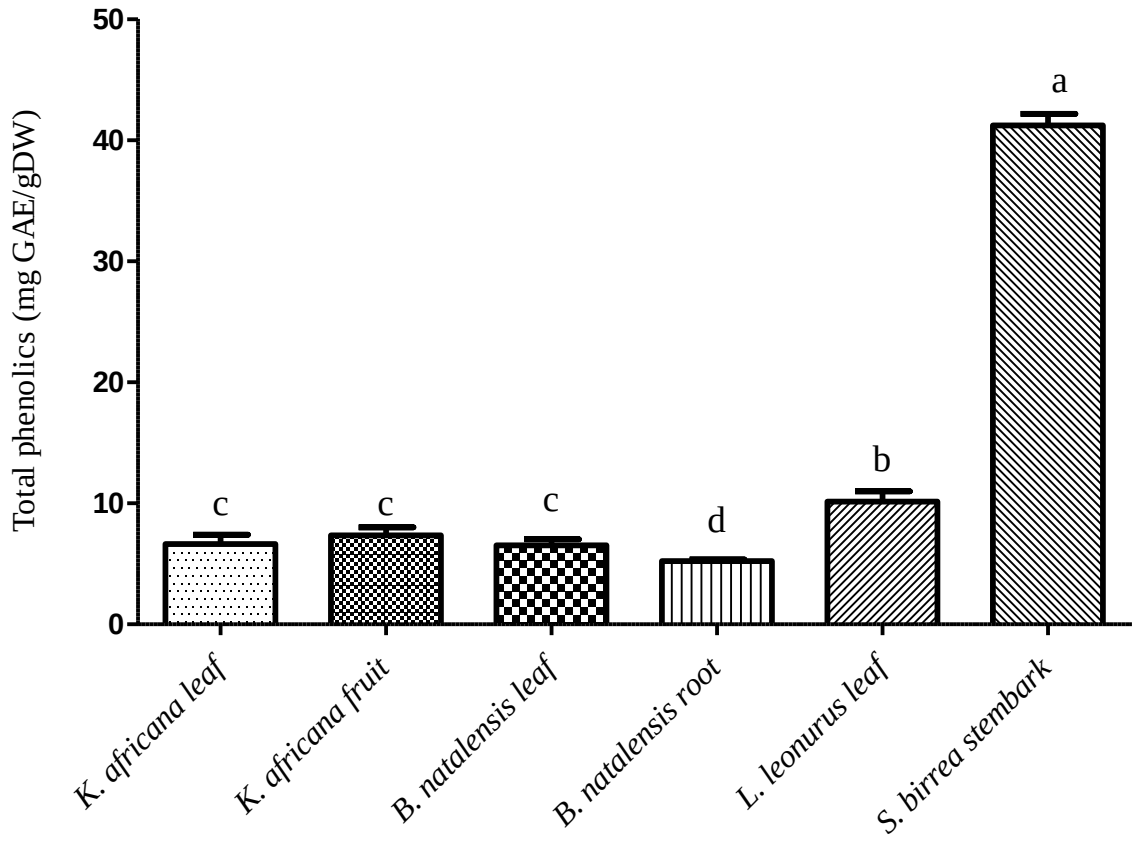

Figure 2. 2: Total phenolic content of selected medicinal plants. Values indicate mean ± SE of three replicates. Different letters between samples indicate significant differences at the 5% level of significance.

2.4.6 Condensed tannins of plant extracts

The results obtained during the determination of quantities of condensed tannins using the butanol-HCL assay are depicted in Figure 2.3. Stem bark of *S. birrea* displayed the highest condensed tannin content (1.91 mg CCE/g DW), while leaves of *K. africana* exhibited the lowest (0.14 mg CCE/g DW).

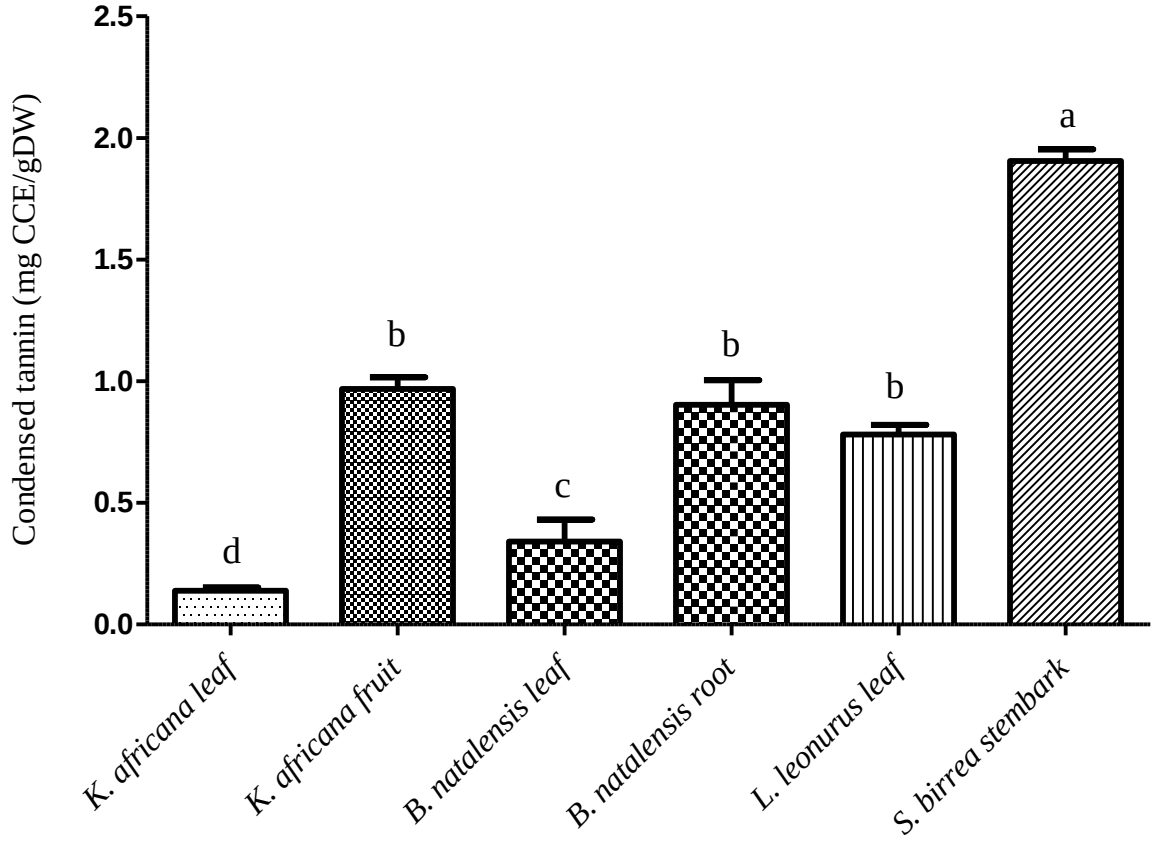

Figure 2. 3: Condensed tannins of selected medicinal plants. Values indicate mean ± SE of three replicates. Different letters between samples indicate significant differences at the 5% level of significance.

2.4.7 Flavonoid content of plant extracts

The flavonoid content of plant extracts is presented in Figure 2.4. The tested plant extracts exhibited varying quantities of flavonoids. *S. birrea* stem bark displayed the highest (175,78

mg CE/g DW) flavonoid content, whereas leaves of *K. africana* (30,08 mg CE/g DW) exhibited the lowest flavonoid content.

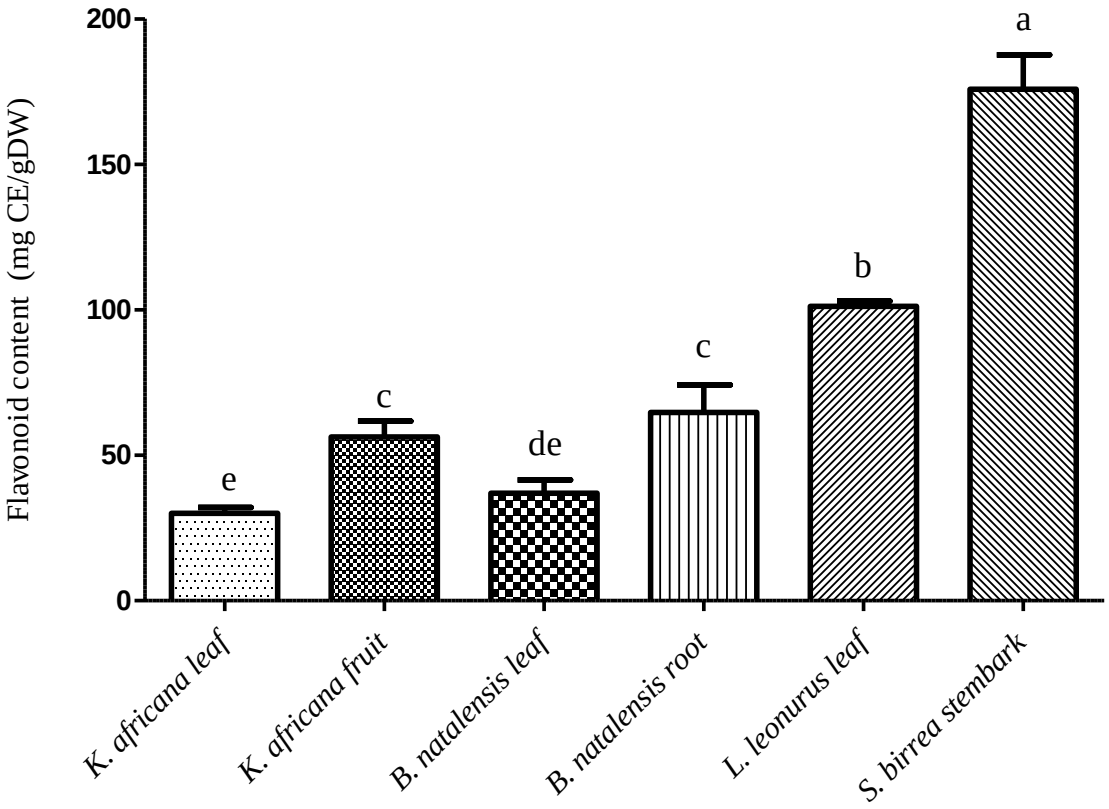

Figure 2. 4: Flavonoid content of selected medicinal plants. Values indicate mean ± SE of three replicates. Different letters between samples indicate significant differences at the 5% level of significance.

2.4.8 Preliminary phytochemical analysis of the investigated plant extracts

Preliminary assessment of the presence of secondary metabolites in extracts (50% MeOH) of the selected medicinal plants revealed the presence of several groups of compounds. All plant extracts were positive for the presence of flavonoids, alkaloids and carbohydrates, however,

the absence of certain groups of compounds was observed in leaves of *B. natalensis* (phenols, terpenoids, tannins and steroids); fruit of *K. africana* (phenols and steroids) and leaves of *K. africana* (steroids).

Table 2. 4: The presence of secondary metabolites in the plants investigated

Plant species	***K. africana***		***B. natalensis***		***L. leonurus***	***S. birrea***
Plant part	**Leaves**	**Fruit**	**Leaves**	**Roots**	**Leaves**	**Stem bark**
Flavonoid	+	+	+	+	+	+
Alkaloid	+	+	+	+	+	+
Terpenoids	+	+	-	+	+	+
Phenols	+	-	-	+	+	+
Tannin	+	+	-	+	+	+
Carbohydrate	+	+	+	+	+	+
Steroid	-	-	-	+	+	+

: - indicates absence

: + indicates presence

Medicinal plants are a rich source of biologically active secondary metabolites such as flavonoids, tannins and phenolic compounds whose bioactivity surpasses that of synthetic agents **(RAVIPATI et al., 2012; VERMA et al., 2013)**. The biological activities of these plants are often attributed to their ability to stabilize or neutralise free radicals due to the

presence of conjugated ring structures, a carboxylic group as well as the nature of their redox properties **(MANDADE et al., 2011; BOLIGON et al., 2014)**. The mechanisms by which these act include the inhibition of the development and scavenging of ROS, offering reducing and metal chelating abilities, inhibiting oxidative enzymes and acting as anti-oxidative enzymes **(BOLIGON et al., 2014)**.

The *in vitro* antioxidant assays revealed that the plant species possess free radical scavenging potential dependent on the type of free radical species present. Heat induced oxidation of β-Carotene and linoleic acid was used to evaluate the ability of plant extracts to delay the bleaching of β-Carotene. The orange β-Carotene emulsion undergoes a swift loss of colour due to a lack of antioxidants because of the production of free radicals within the system **(AMAROWICZ et al., 2004; MARIOD et al., 2010)**. The presence of antioxidants can delay the rate of β-Carotene bleaching by stabilizing the linoleate free radical as well as several other free radicals that may be formed in the system **(AMAROWICZ et al., 2004; MARIOD et al., 2010)**. Investigation of plant extracts based on this model revealed a wide range of antioxidant activities ranging from very poor to potent. The ACE and water extracts of the stem bark of *S. birrea* demonstrated very high antioxidant activity. Other plant extracts which had notable antioxidant activity include, leaves of *L. leonurus* (water); roots of *B. natalensis* (ACE and water) and leaves of *K. africana* (water). It was also noted that PE extracts of leaves of *L. leonurus* and stem bark of *S. birrea* did not display any form of activity against β-Carotene bleaching. The leaves and roots of *B. natalensis* (all extracts, PE and DCM, respectively), leaves and fruit of *K. africana* (organic), leaves of *L. leonurus* (DCM and ACE) as well as stem bark of *S. birrea* (DCM) displayed very weak antioxidant activity, of which most demonstrated an inability to limit the bleaching of β-Carotene after 120 min. Despite the presence of phenolic

compounds in these plants, the results contrast with **LEONTOWICZ et al. (2003)**, who reported a direct correlation of polyphenolic content and BCA activity in apple peel and pulp. However, **MARIOD et al. (2010)**, reported that there is no correlation between the phenolic content of plants and their BCA activity, this was also reported by **AMAROWICZ et al. (2004)**. Furthermore, the antioxidant activities of plant extracts cannot be anticipated based on the total phenolic content because the response of antioxidant activity with phenolic compounds on lipid substrates is greatly dependent on chemical structure and oxidation conditions **(AMAROWICZ et al., 2004)**.

All plant extracts demonstrated different levels of propensity to reduce DPPH free radicals. This action occurs through electrons or hydrogen atom donation, thereby causing a rapid decrease in the absorbance of the reaction mixture in the presence of the plant extract **(AMAROWICZ et al., 2004; BOLIGON et al., 2014)**. DPPH is a molecule containing a stable free radical which is reduced by antioxidant compounds and causes decay of the purple chromogen DPPH to a pale-yellow hydrazine **(BOLIGON et al., 2014)**. The DPPH molecule can delocalise additional electrons over the entire molecule thus preventing the molecule to dimerize as would other free radicals **(ALAM et al., 2013)**. The results obtained in this experiment revealed a varied ability of plant extracts (organic and water) to influence DPPH radical scavenging. **OYEDEMI and AFOLAYAN (2011)** reported *L. leonurus* to be a strong DPPH reducer and the results obtained in this study (Table 2.3), displayed by the DCM, ACE and water extracts agree with these findings. Moreover, the isolated compound known as 'marrubiin' found in *L. leonurus* is believed to be associated with its bioactivities **(NSUALA et al., 2015)**. Extracts of stem bark of *S. birrea* (ACE and water) as well as the leaves (DCM and water) and fruit (ACE and water) of *K. africana*, demonstrated strong free radical scavenging activity which was comparable to that of the standard control (ASC and BHT). The

observed antioxidant activity of *S. birrea* stem bark in this study concurs with those of **MASOKO et al. (2008)** who reported antioxidant activity of leaves and rhizomes extracts (dichloromethane, hexane, and acetone). Furthermore, **MOYO et al. (2010)** also reported radical scavenging activity of young stems of *S. birrea* and the stem bark of *Harpephyllum caffrum*. The observed activity of the stem bark of *S. birrea* may be due to the direct relationship between the total phenolic content and the antioxidant activity demonstrated **(AMAROWICZ et al., 2004; MOYO et al., 2010)**. The ACE leaves and PE fruit extracts of *K. africana* displayed very weak antioxidant activity (18 and 8%, respectively) in accordance with **AKANNI et al. (2014)**, who reported weak DPPH activity from fruit of *K. africana*. Contrastingly, **DHRITI et al. (2014)** reported good antioxidant activity from ACE leaf extracts of *K. africana* (65%), moreover **OLUBUNMI et al. (2009)**, reported that the wax removed from the leaves of *K. africana* displayed very high free radical scavenging activity which is in accordance with the results obtained from leaves and fruit extracts of *K. africana* (DCM and water; ACE and water, respectively). These contrasting findings may be attributed to geographic variation in phytochemicals and different methods of extraction. The observed overall inhibitory potential of the plant extracts against the DPPH radical displayed a range of IC_{50} values (0.01 to 0.22 mg/mL) (Table 2.3), which are directly linked to their antioxidant activities indicating their potential as proton donors and possible oxidative stress relievers.

PATEL et al. (2013) reported that hyperglycemia causes augmented productions of hydrogen peroxide, which could result in RNA, DNA and lipid impairment. H_2O_2 is capable of infiltrating biological membranes and is thus considered a highly reactive oxygen species **(CHEN et al., 2012)**. It is rapidly converted into oxygen and water by a CAT reaction which neutralises it, however in the absence of CAT, it may result in the production of hydroxyl radicals (OH^-) which may induce lipid peroxidation **(CHEN et al., 2012; ASMAT et al.,**

2016). It is therefore of importance for antioxidants to remove H_2O_2 in cells as well as food products to prevent cellular damage **(KESER et al., 2012)**. The antioxidant activity (Table 2.3) of ACE extracts of roots of *B. natalensis*; fruit of *K. africana*; stem bark of *S. birrea* not only displayed strong antioxidant activities but also had higher IC_{50} values, which where greater than those of the reference drugs. The different water extracts of the plants also showed a similar trend. These findings concur with **KIBITI and AFOLAYAN (2015)** who reported that the ACE and water extracts of the *B. absyssinica* showed higher antioxidant activity than the standard drugs. Therefore, the obtained results indicate that the bioactive compounds present in these plants have the potential to transfer electrons to OH^-, produced by H_2O_2 neutralising it to water **(LOBO et al., 2010; ASMAT et al., 2016)**.

The ferric reducing potential of extracts of the different plant species used in this research was determined by measuring the reduction of Fe^{+3} to Fe^{+2}. The results revealed that the plant extracts possessed satisfactory reducing abilities which were concentration dependent (Figure 2.1). The stem bark of *S. birrea* demonstrated high phenolic content and displayed strong reducing power, which was generally higher than that of the standard control (BHT). Several researchers have reported the direct relationship between the ferric reducing potentials of plant extracts with phenolic content compounds **(MOYO et al., 2010)**. *B. natalensis* demonstrated moderate to weak ferric reducing power but this is contrary to what has been reported by **SAGBO and MBENG (2018)**, who reported that the extract of *B. latifolia* demonstrated strong reducing powers, greater than the standard control BHT. The weak antioxidant activity demonstrated by *B. natalensis* concurs with the results of **MOSA et al. (2011)**, who reported that *B. natalensis* is a poor antioxidant agent. The activity of these plant extracts was distinguished by the formation of Perl's Prussian blue colouration after ionic reduction of the ferric ion/ferricyanide complex to a ferrous form. This conversion is a result of the reaction

with ROS, thus converting them to a more stable product and halting the radical chain reaction **(NDHLALA et al., 2014)**. The reducing activity of these plant extracts could be linked to their ability to donate electrons due to present metabolites **(SHARMA et al., 2012)**.

The results obtained in the present study indicate that the selected medicinal plant species contain alkaloids, flavonoids, carbohydrates, terpenoids, tannins and steroids (Table 2.4) as well as considerable quantities of total phenolics, condensed tannins and flavonoids (Figure 2.2; 2.3 and 2.4, respectively). According to **BRASILEIRO et al. (2015)**, numerous researchers have demonstrated that phenolic content in plants may vary based on growth stage, plant organs as well as environmental conditions. Biologically active compounds such as tannins, quinones, saponins, alkaloids, phenolics and terpenoids have previously been identified in *L. leonurus* **(OYEDEMI and AFOLAYAN, 2011; LAONIGRO et al., 1979; DEMETZOS and DIMAS, 2001)**. Furthermore, the stem bark of *S. birrea* demonstrated the highest phenolic content. This is in accordance with **MOYO et al. (2010)** who reported high phenolic content in young stem extracts of *S. birrea*. The phenolic content of the stem bark of *S. birrea* could contribute to the superior antioxidant activity observed in this study. Phenolics and flavonoids are the most frequent compounds found in the majority of plants that have been reported to possess free radical scavenging potentials (**KIBITI and AFOLAYAN, 2015; AZWANIDA, 2015)**.

2.5 Conclusions

Some of the plants extracts under investigation have demonstrated the presence of secondary metabolites and their abilities to act as free radical scavengers. Therefore, these plant species may be used to prevent the induction and spread of oxidative chain reactions thereby averting

or delaying diseases such as diabetes and diabetic related complications **(OYEDEMI and AFOLAYAN, 2011)**.

CHAPTER THREE: *IN VITRO* ANTIDIABETIC AND ANTIMICROBIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS

3.1 Introduction

Diabetes mellitus (DM) is a multifaceted chronic disorder distinguished by hyperglycemia **(RENGASAMY et al., 2013)**. Hyperglycemia has been reported as a possible cause of the non-enzymatic glycosylation of several macromolecules including the mutation of antioxidants and/or production of free radical species. Moreover, chronic hyperglycemia may cause damage in cells so they are unable to regulate the absorption of sugar **(LEBOVITZ, 2001; McCUE and SHETTY, 2004; MARTINEZ et al., 2005)**. A feasible approach to significantly lower hyperglycemia, may be through preventing or slowing down the absorption of carbohydrate hydrolysing enzymes (i.e. α-amylase and α-glucosidase), postprandialy (**ADEMILUYI and OBOH, 2013; ORTIZ-ANDRADE et al., 2007)**.

The α-amylase carbohydrate enzyme hydrolyses complex polysaccharides by releasing glucose through the intestinal α-glucosidase prior to absorption in the gut and circulatory system **(ADEMILUYI and OBOH, 2013).** α-Glucosidase is a vital enzyme responsible for promoting intestinal glucose absorption through oligosaccharides and disaccharides cleaving them into simple sugars (**CHUKWUJEKWU et al., 2016; RENGASAMY et al., 2013)**. Primarily, α-amylase inhibitors utilise their hypoglycemic effects by inhibiting salivary and pancreatic amylase and are therefore recognised as starch blockers **(KAZEEM et al., 2013)**. Furthermore, increased blood glucose levels postprandial may be subdued by α-glucosidase inhibitors which could slow down and prolong carbohydrate digestion and digestion time respectively, through the inhibition of α-glucosidase in the intestine **(KAZEEM et al., 2013)**. Synthetic agents such as voglibose, acarbose and miglitol are broadly utilised as inhibitors of carbohydrate

hydrolysing enzymes in Type 2 DM, but they produce undesirable side effects such as diarrhoea and flatulence **(LEBOVITZ, 1997)**.

Infectious diseases are identified as maladies caused by microorganisms such as fungi, bacteria, viruses or parasites. These organisms are responsible for the increased human mortality rates worldwide. For instance, **MORENS et al. (2004)** reported that of the 57 million annual deaths occurring globally, 25% are associated with infectious diseases.

Diabetic individuals are vulnerable to infections as a result of their compromised and weakened immune systems, caused by chronic hyperglycemia. Moreover, diabetes related complications such as nephropathy and atherosclerosis, increase susceptibility to infections in patients **(ROSS, 2018)**. In most cases, diabetic patients are more prone to infectious diseases such as tuberculosis (TB) and pneumonia **(CASQUEIRO et al., 2012)**. The most prevalent infections include foot infections provoked by microorganisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis* and urinary tract infections (UTIs) mainly caused by uropathogens such as *Escherichia coli* and *Enterococcus faecalis*. Fournier gangrene affects 70% of diabetic males and is triggered by *E. coli* and *Klebsiella* sp. **(CASQUEIRO et al., 2012)**. Fungal infections such as rhinocerebral mucormycosis, occurs in approximately 50% of DM patients and is considered a deadly infection that is promoted by *Mucor* or *Rhizopus*. Yeast infections such as fungal cystitis, oral and esophageal candidiasis are also frequent, being caused by several species of the genus *Candida* (**de LEON et al., 2002; MOHAMMADI et al., 2016; CASQUEIRO et al., 2012; ROSS, 2018)**. These infections may exacerbate diabetic related complications such as hypoglycemia and ketoacidosis **(PELEG et al., 2007)**.

Medicinal plants are reported to possess antidiabetic and antimicrobial properties, owing to the presence of a broad range of bioactive compounds **(JOSEPH and JINI, 2013)**. Conventional medicines (i.e. acarbose, antibiotics or fungicides) used to treat these ailments are costly, often

inaccessible and may cause adverse side effects. Additionally, multi-drug resistance by pathogens also make it difficult, not only to clinically manage hyperglycemia but also to treat associated infections **(MISHRA et al., 2007)**. These difficulties have encouraged the use of holistic therapies from plants. Therefore, this study was designed to investigate and evaluate the antidiabetic and antimicrobial properties of the selected medicinal plants.

3.2 Materials and Methods

3.2.1 Extract preparation

Extract preparation prior to the execution of the biological experiments were prepared as described in Chapter 2, section 2.2.2. The organic (PE, DCM and ACE) and aqueous water extracts were dissolved at a concentration of 0.5 dimethyl sulfoxide (DMSO) and 10 (20% DMSO) mg/mL for enzyme and antimicrobial assays, respectively.

3.2.2 *In vitro* antidiabetic assays

3.2.2.1 α-Amylase inhibition assay

Inhibition of α-amylase assay was performed with the use of the 3.5-dinitrosalicylic acid (DNSA) method described by **WICKRAMARATNE et al. (2016)**, with minor modifications. Sodium phosphate buffer (0.02 M; SPB) containing 6.7 mM NaCl (pH 6.9), was used to prepare α-amylase (from *Aspergillus oryzae*, Sigma-Aldrich) enzyme solution (2 U/mL). The sample extracts were dissolved in 10% DMSO and further dissolved with the SPB to a range of concentrations (0.01-0.1 mg/mL). To a test tube, 200 µL each of the extract and the enzyme solution were added. The mixture was pre-incubated for 10 min in a water bath at 30 $^{\circ}$C. Post pre-incubation, 200 µL of the substrate starch solution (1% in phosphate buffer w/v) was added to each tube to start the reaction, which was further incubated for 3 min in a water bath at 30 ^{o}C. The reaction was terminated by addition of 200 µL of DNSA coloring reagent (12 g of

sodium potassium tartate tetrahydrate in 8 mL of 2 M sodium hydroxide and 20 mL of 96 mM of 3.5-dinitrosalicylic acid solution) and boiled in a water bath at 85 ºC for 10 min. The mixture was allowed to cool, and distilled water was added to each test tube to a final volume of 5 mL. The absorbance was read at 540 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). Sodium phosphate buffer was used to blank the spectrophotometer. The negative control was prepared by replacing sample extract with 200 µL of buffer (100% enzyme activity). Reaction control was prepared identical to that of sample extract for each concentration, however, the enzyme solution was replaced by sodium phosphate buffer (200 µL). Acarbose (0.1 mg/mL) was prepared in the same manner as the sample extract reaction, as mentioned above and used as a reference drug. Percentage inhibition of the enzyme was calculated as follows:

$$\% \text{ α-amylase inhibition} = 100 \times Abs_{Control} - Abs_{Sample} / Abs_{Control}$$

Abs is the absorbance of either control or sample

3.2.2.2 α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity of the extracts was determined as described by **TAO et al. (2013)** with modifications as described by **RENGASAMY et al. (2013)**, Potassium phosphate buffer (0.1M, pH 6.8; PPB) was used to prepare a α-glucosidase (from *Saccharomyces cerevisiae*, Sigma-Aldrich) enzyme solution (0.1 U/mL) and the 0.375 mM *p*-nitrophenyl-*a*-D-glucopyranoside substrate solution. Sample extracts were dissolved in DMSO to known concentrations from (0.1- 0.00625 mg/mL) independently. In a 96-well microtiter plate, 20 µL of each sample extract were added into the wells in triplicate and serially diluted using a multichannel pipette. To the extract solution, 20 µL of enzyme solution was added and the mixture was pre-incubated for 5 min at 37 ºC. To initiate the reaction, the substrate solution (40 µL) was added to the reaction mixture and further incubated for 30 min at 37 ºC. Sodium

carbonate (0.2 M; 80 µL) prepared in PPB was then added to each well of the mixture to stop the reaction. The quantity of *p*-nitrophenyl produced by the reaction as indicated by the intensity of change in colour (yellow) during the reaction was determined using an Opsys MR 96-well microplate reader at 405 nm. Acarbose dissolved in DMSO (60 mg/mL) and treated in the same way was used as reference drug. The phosphate buffer instead of the sample extract was used as the control. The percentage inhibition of the enzyme was calculated as follows:

$$\% \text{ α-glucosidase inhibition} = 100 \times Abs_{Control} - Abs_{Sample} / Abs_{Control}$$

Abs is the absorbance of either control or sample

3.2.3 Antimicrobial assays

3.2.3.1 Antibacterial micro-dilution assay

Sample extracts were evaluated for antibacterial activity in the microdilution assay as described by **ELOFF (1998)**, where the minimum inhibitory concentrations (MIC) were determined. Bacterial cultures including two Gram-positive (*Enterococcus faecalis* ATCC 19433 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) bacterial species were incubated (37 ºC) overnight in a water bath with an orbital shaker. The respective bacterial strains were diluted with 20 mL sterile Mueller-Hinton (MH) broth (200 µL in 19.8 mL) to a final inoculum concentration of 10^6 CFU/mL. Crude organic extracts were suspended in 20% DMSO and water extracts were dissolved in sterile water, to a final concentration of 10 mg/mL. In a 96-well microplate, 100 µL of each sample extracts were serially diluted two-fold with 100 µL of sterile distilled water. One hundred microlitres of bacterial strain was then added separately to each of the extract

wells. The microplates were covered with parafilm and incubated at 37 ºC for 24 h. A two-fold serial dilution of neomycin (Sigma-Aldrich, Steinheim, Germany) (0.1 mg/ml) was used as a positive control against each bacterial strain while water was used as the negative control and 20% DMSO as the solvent control. Subsequently, an indicator, p-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) (40 µL 0.2 mg/ml) was added to each well to determine MIC values after an incubation period of 1 h. The presence of biologically active organisms is indicated by the tetrazolium salt which results in a red product. The lack of a colour change indicates inhibition of the test organism by the plant extracts. The bioassay was performed with three replicates per sample extract or control and repeated twice.

3.2.3.2 Antifungal micro-dilution assay

The antifungal activity of the extracts was determined using the method described by **ELOFF (1998)**. An overnight culture of *Candida albicans* (ATCC 10231) in a yeast malt (YM) broth was incubated in a water bath with an orbital shaker (37 °C). To 4 ml of sterile saline, 400 µL of the fungal culture was added and the absorbance read and adjusted at 530 nm, to match that of the 0.5 M McFarland standard solution from which a 1:1000 dilution was prepared with sterile YM broth, producing a final inoculum of approximately 10^6 colony-forming units (CFU/mL). The organic sample extracts were suspended in 20% DMSO while the water extracts were dissolved in sterile water to a final concentration of 10 mg/mL. In a 96-well microplate, 100 µL of each extract were serially diluted two-fold with 100 µL of sterile distilled water. One hundred microlitres of the diluted fungal culture was subsequently added to each

well. Amphotericin A (Sigma-Aldrich, Germany, 2.5 mg/mL) was used as positive control, and a similar two-fold serial dilution was used while water and 20% DMSO served as negative and solvent controls, respectively. The microplates were then covered with parafilm and incubated at 37 ºC for 24 h. Post incubation, 40 µL 0.2 mg/ml INT was added from which the plates were further incubated for another 24 h at 37 ºC. Clear wells indicated inhibition of fungal activity while reddish-pink wells indicated fungal growth. The MIC values of extracts that inhibited fungal growth were recorded. The bioassay was performed with three replicate sample extracts and repeated twice.

3.2.4 Statistical analysis

The obtained enzyme data of the plant extracts were subjected to a One-way-analysis of variance (ANOVA) using IBM SPSS software version 21 for Windows. Significant differences ($p = 0.05$) of the means were separated using Duncan's Multiple Range Tests and IC_{50} values were conducted on GraphPad Prism Version 4.00 for Windows (GraphPad, Software Inc.)

3.3 Results and Discussion

3.3. 1 α-Amylase inhibitory activity of plant extracts

Plant extracts were measured for their ability to inhibit the α-amylase enzyme responsible for starch breakdown in the gut (Figure 3.1). The inhibitory activity of the extracts at 0.1 mg/mL, were higher for DCM and water extracts of leaf extracts of *B. natalensis* (71.13 and 79.45%, respectively), water extract of roots of *B. natalensis* (89.53%), ACE extract of leaves of *L. leonurus* (73.54%), and water extracts of leaves of *K. africana* and *S. birrea* stem bark (75.16 and 70.65%, respectively) than that of acarbose (64.47%). Moderate inhibitory activity was displayed by PE and ACE extracts of leaves of *K. africana* (41.13 and 47.39%, respectively) and ACE roots (33.41%) and PE leaves (36.21%) of *B. natalensis*, water extracts of *L. leonurus* (32.40%) and DCM and ACE extracts of stem bark of *S. birrea* (32.40 and 33.81%, respectively). The IC_{50} values presented in Table 3.1, ranged from 0.01 to 0.07 mg/mL, with the water extracts of leaves and fruit of *K. africana* (0.01 and 0.02 mg/mL), PE extracts of leaves of *K. africana* (0.03 mg/mL), water extracts of leaves of *B. natalensis* (0.03 mg/mL) and also water extracts of *S. birrea* stem bark (0.02 mg/mL) displayed low IC_{50} values which were better and sometimes comparable to that of the standard control (0.03 mg/mL).

1. *K. africana* L
2. *K. africana* F
3. *B. natalensis* L
4. *B. natalensis* R
5. *L. leonurus* L
6. *S. birrea* SB

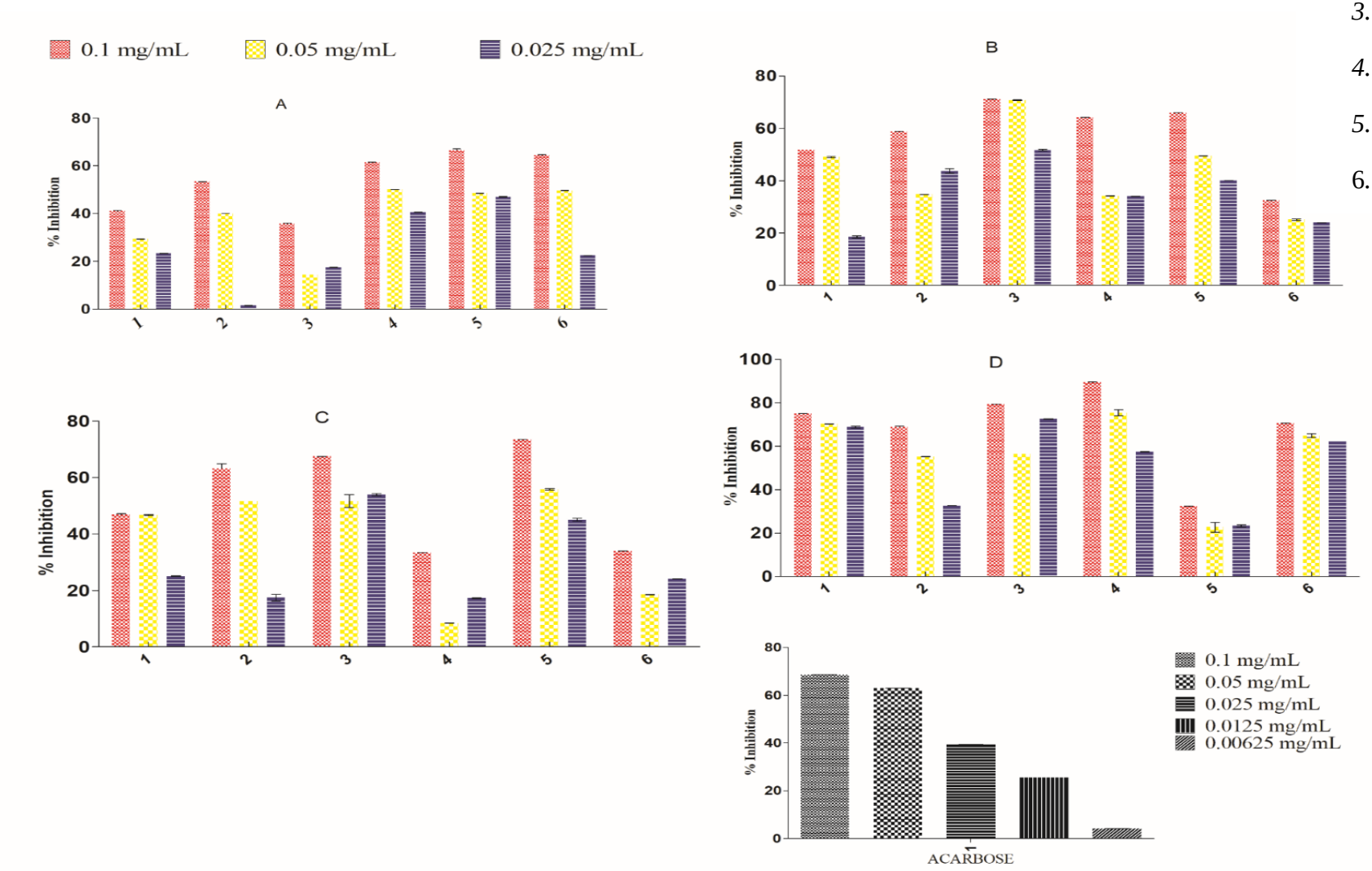

Figure 3. 1: α-amylase inhibitory activity of plant extracts. F= fruit, L= leaves, R= roots, SB= stem bark. Extract solvents: A= petroleum ether, B= dichloromethane, C= acetone, D= water. Values indicate mean ± SE of three replicates. SE indicate significant differences at the 5% level of significance.

Table 3. 1: IC_{50} values of the α-Amylase and α-Glucosidase activity of extracts

			IC_{50} mg/mL	
Plant species	**Plant part**		**α-Amylase**	**α-Glucosidase**
Bulbine natalensis	Leaves	PE	0.07±0.000[a]	0.05±0.003[a]
		DCM	0.04±0.001[efg]	0.05±0.012[a]
		ACE	0.04±0.003[ghi]	0.06±0.001[a]
		Water	0.03±0.000[jk]	0.06±0.011[a]
	Roots	PE	0.04±0.003[de]	0.05±0.005[a]
		DCM	0.06±0.000[b]	0.04±0.007[a]
		ACE	0.05±0.000[c]	0.05±0.002[a]
		Water	0.04±0.001[ij]	0.06±0.010[a]
Kigelia africana	Leaves	PE	0.03±0.000[l]	0.06±0.001[a]
		DCM	0.05±0.000[c]	0.07±0.001[a]
		ACE	0.04±0.001[ghij]	0.05±0.004[a]
		Water	0.01±0.000[m]	0.04±0.013[a]
	Fruit	PE	0.05±0.000[d]	0.05±0.004[a]
		DCM	0.04±0.003[efg]	0.05±0.006[a]
		ACE	0.04±0.000[hij]	0.04±0.002[a]
		Water	0.02±0.000[n]	0.03±0.016[a]
Leonotis leonurus	Leaves	PE	0.06±0.000[b]	0.04±0.003[a]
		DCM	0.05±0.000[c]	0.03±0.007[a]
		ACE	0.04±0.003[def]	0.04±0.010[a]
		Water	0.04±0.002[efg]	0.05±0.014[a]
Sclerocarya birrea	Stem bark	PE	0.04±0.000[fgh]	0.05±0.002[a]
		DCM	0.04±0.001[fgh]	0.05±0.008[a]
		ACE	0.06±0.002[b]	0.02±0.001[a]
		Water	0.02±0.000[m]	0.03±0.008[a]
Acarbose			0.03±0.000[kl]	1.20±0.027[b]

Values indicate mean ± SE of three replicates. PE= petroleum ether, DCM= dichloromethane, ACE= acetone. Different letters in the same column indicate significant differences at the 5% level of significance

3.3.2 α-Glucosidase inhibitory activity of plant extracts

The potential of the plant extracts to inhibit the α-glucosidase enzyme was measured (Figure 3.2). High inhibitory activity of the extracts at 0.1 mg/mL, was observed from DCM and ACE extracts of roots of *B. natalensis* (98.84 and 96.42%, respectively), ACE extracts of leaves of *B. natalensis* (72.49%), ACE extracts of fruit of *K. africana* (84.23%) and ACE and water extracts of *S. birrea* stem bark (94.04 and 95.53%). Weak to moderate inhibitory activity was observed from all water extracts except for *S. birrea* (95.53%). All IC_{50} values of the extracts ranged from 0.02 to 0.07 mg/mL which were significantly lower than the standard control acarbose (1.20 mg/mL).

2. *K. africana* L

2. *K. africana* F

3. *B. natalensis* L

4. *B. natalensis* R

5. *L. leonurus* L

6. *S. birrea* SB

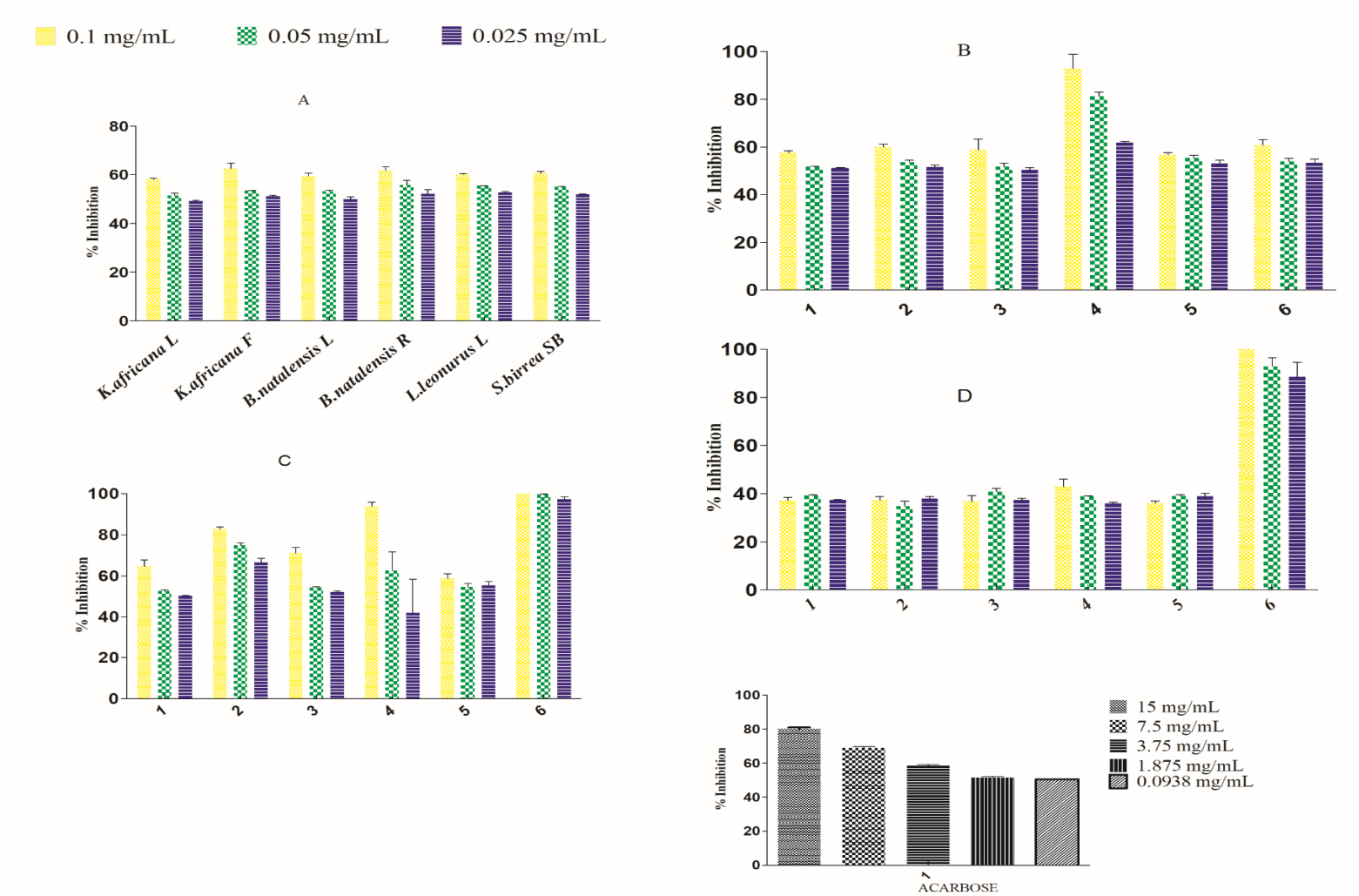

Figure 3. 2: α-glucosidase inhibitory activity of plant extracts. F= fruit, L= leaves, R= roots, SB= stem bark. Extract solvents: A= petroleum ether, B= dichloromethane, C= acetone, D= water. Values indicate mean ± SE of three replicates. SE indicates significant differences at the 5% level of significance

3.3.3 Antibacterial and antifungal activity of plant extracts under investigation

The minimum inhibitory concentration (MIC) values (antibacterial and antifungal) of the plant extracts are presented in Table 3.2. Twenty-four plant extracts of the selected plant species were screened for antimicrobial activity. The results suggest that some of the investigated plant extracts were able to inhibit the pathogenic strains, while others were inconclusive and required a concentration above that of the highest tested concentration (Table 3.2). Broad spectrum antimicrobial inhibitory activity against both the bacterial and fungal strains was demonstrated by the DCM extract of roots of *B. natalensis*, ACE and water extract of *S. birrea* stem bark and ACE extracts of leaves of *L. leonurus*. In addition, the DCM extract of leaves of *B. natalensis* and the ACE extract of roots of *B. natalensis* displayed effective microbial inhibitory activity against all bacterial strains used. However, water extracts of leaves and roots of *B. natalensis*, leaves of *K. africana* and *L. leonurus*, showed no noteworthy antimicrobial activity.

Table 3. 2: Antibacterial and antifungal activity (MIC mg/mL) of extracts.

			S.a	E.f	E.c	K.p	C.a
Plant species	**Plant part**						
Bulbine natalensis	Leaves	PE	**0.63**	**0.63**	**0.02**	2.50	2.50
		DCM	**0.31**	**0.31**	**0.02**	**0.31**	1.25
		ACE	1.25	1.25	2.5	1.25	1.25
		Water	>2.50	2.50	>2.50	>2.50	>2.50
	Roots	PE	>2.50	>2.50	>2.50	>2.50	2.50
		DCM	**0.63**	**0.63**	**0.63**	**0.16**	**0.16**
		ACE	**0.16**	**0.16**	**0.02**	**0.16**	>2.50
		Water	1.25	>2.50	>2.50	>2.50	>2.50
Kigelia africana	Leaves	PE	**0.31**	1.25	**0.02**	2.50	2.50
		DCM	**0.31**	**0.31**	**0.02**	>2.50	2.50
		ACE	**0.63**	**0.63**	**0.02**	2.50	2.50
		Water	>2.50	>2.50	2.50	2.50	>2.5
	Fruit	PE	>2.50	>2.50	>2.50	>2.50	2.50
		DCM	>2.50	>2.50	>2.50	1.25	>2.50
		ACE	**0.63**	**0.31**	**0.63**	>2.5	>2.50
		Water	**0.63**	**0.63**	>2.5	>2.5	>2.50
Leonotis leonurus	Leaves	PE	1.25	2.50	**0.63**	2.50	**0.02**
		DCM	2.50	**0.08**	**0.63**	2.50	1.25
		ACE	**0.63**	**0.31**	**0.63**	**0.31**	**0.02**
		Water	>2.50	>2.50	>2.50	>2.50	>2.50
Sclerocarya birrea	Stem bark	PE	>2.50	>2.50	>2.50	>2.50	2.50
		DCM	2.50	>2.50	1.25	2.50	2.50
		ACE	**0.16**	**0.08**	**0.02**	**0.16**	**0.16**
		Water	**0.31**	**0.31**	**0.63**	**0.63**	**0.04**
Neomycin (µg/mL)			0.78	0.63	0.16	0.16	
Amphotericin B (µg/mL)							0.93

Values indicate mean of three replicates. PE= petroleum ether, DCM= dichloromethane, ACE= acetone, S.a= *Staphylococcus aureus*, E.f= *Enterococcus faecalis*, E.c= *Escherichia coli*, K.p= *Klebsiella pneumoniae*, C.a= *Candida albicans*. Values in bold are considered very active (MIC ≤ 1 mg/mL).

The management of DM is a major global challenge with effective therapies still to be discovered **(KAZEEM et al., 2013)**. Early metabolic impairments identified from diabetic patients is displayed by increased postprandial hyperglycemia **(BLAAK et al., 2012)**. The critical approach in the management of DM involves achieving the control of postprandial hyperglycemia which, if left untreated could lead to the development of microvascular and macrovascular complications **(CHANG et al., 2013; ADEMILUYI and OBOH, 2013)**.

Frequently used hypoglycaemic medicines in the treatment of T2DM include α-glucosidase inhibitors such as acarbose. Acarbose is a nitrogen-containing pseudo-tetrasacharride, forming part of the α-glucosidase inhibitor group **(SHIBAO, 2012)**. However, acarbose has been reported to exhibit very little to no inhibitory activity against α-glucosidase hence the upsurge of interest in the search for more effective treatments **(ANAM et al., 2009; SHAI et al., 2010)**. Medicinal plants and herbal therapies have been marked as efficient approaches to inhibiting the major DM associated enzymes (α-amylase and α-glucosidase) to reduce the level of blood glucose after a meal **(ADEMILUYI and OBOH, 2013; KONATE et al., 2014**). As part of their defence mechanisms plants produce a wide variety of compounds, some of which are glucosidase inhibitors and may be effective in the suppression of hyperglycemia **(KAVIMANI et al., 2014)**.

The *in vitro* antidiabetic assays demonstrated the inhibitory activity of the selected plant species. The inhibition of the pancreatic α-amylase enzyme may reduce the breakdown of starch in the small intestine, thereby lowering plasma glucose levels **(SARAVANAN and PARIMELAZHANGAN, 2014)**. The results of the present study (Figure 3.1) revealed the strong inhibitory activity of leaf extracts of *B. natalensis* (DCM and water), leaves of *L. leonurus* (ACE), leaves of *K. africana* (water) and *S. birrea* stem bark (PE and water). A study by **KAZEEM et al. (2013)**, reported high α-amylase inhibitory activity by leaf extracts of *Morinda lucida*. Furthermore, potent α-amylase inhibition by plant extracts have been reported

previously to lead to irregular bacterial fermentation of undigested carbohydrates in the gut, therefore only moderate α-amylase inhibition is desirable **(KAZEEM et al., 2013)**. Mild inhibitory activity was demonstrated by leaves of *K. africana* (PE and ACE), leaves of *B. natalensis* (PE), roots of *B. natalensis* (DCM and ACE), leaves of *L. leonurus* (water) and stem bark of *S. birrea* (DCM and ACE). **DHRITI et al. (2014)**, reported a significant reduction in α-amylase activity from the ethanolic leaf extract of *K. africana*, which concurs with the obtained findings. The α-amylase inhibitory activities of the plant extracts may be due to the presence of secondary metabolites such as tannins and flavonoids. Phytochemicals such as phenolic acids and flavonoids are reported to be covalently attached to the α-amylase and alter its activity because of the capacity to develop quinones or lactones which can react with nucleophilic groups found on the enzyme **(OYEDEMI et al., 2013)**.

Potent α-glucosidase inhibitory activity (Figure 3.2) was displayed by roots of *B. natalensis* (DCM and ACE, respectively), leaves of *B. natalensis* (ACE), fruit of *K. africana* (ACE) and *S. birrea* stem bark (ACE and water). These results conform with those of **KWON et al. (2006)**, who reported that natural *α*-glucosidase inhibitors from plants have been shown to exhibit strong inhibitory activity against *α*-glucosidase i.e. (rosemary and sage) and thus could serve against postprandial hyperglycemia with minimal side effects. The potent inhibitory activity of *S. birrea* corroborates with those reported by **DIMO et al. (2007)**, who showed that dichloromethane: methanol (1:1) extracts could decrease hyperglycemia while increasing plasma insulin levels in streptozotocin (STZ) rats. Additionally, a study done by **VAN DE VENTER et al. (2008)**, revealed that a methanol extract of *S. birrea* led to a notable rise of glucose use in Chang liver cells. The reported bioactivity of ACE and aqueous extracts of *S. birrea* may be due the presence of phytochemicals such as terpenes and flavonoids, which have been reported to exhibit hypoglycaemic activities **(PATEL et al., 2012)**. **PRIYA et al. (2014**)

reported a methanolic leaf extract of *K. africana* was found to reduce serum glucose levels in alloxan-induced diabetic rats. Although the leaf extracts of *L. leonurus* in this study demonstrated moderate to weak inhibitory activity, **OYEDEMI et al. (2011)** demonstrated the hypoglycemic potential of the water extracts of the leaf of *L. leonurus* as well as it's antilipidemic effects in STZ-induced diabetic rats. Several secondary metabolites were noted from the evaluated plant species (Chapter 2). Secondary metabolites generally play vital roles in phytomedicines, for instance they are known to stimulate and regulate the liberation of insulin in the pancreas for glucose uptake by the muscle tissue (**VIRGINIE et al., 2016)**. Alkaloids have also been reported to possess anti-hyperglycemic activities because of their ability to inhibit and reduce α-glucosidase and glucose transport through the intestinal lining **(PAN et al., 2003)**. Additionally, flavonoids have also been known to regenerate injured β-cells in diabetic rats, therefore they are also considered effective hypoglycemic agents **(CHAKRAVARTHY et al., 1980; MANICKAM et al., 1997)**.

The PE and DCM extracts of leaves of *B. natalensis* displayed good (0.63-0.02 mg/mL) bioactivity against the Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Klebsiella pnuemoniae*) bacteria. No noteworthy activity from the ACE and water extracts were demonstrated. In addition, the roots of *B. natalensis* (DCM and ACE) showed potent bioactivity against all the tested bacterial strains, however, PE and water extracts displayed reduced antibacterial activity against the pathogens. These findings concur with **YAKUBU et al. (2012)**, who reported noteworthy antibacterial activity of ethanol *B. natalensis* tuber extracts against several pathogens including (*S. aureus, E. coli* and *K. pneumoniae*). The human intestinal flora contains *E. coli* which could cause diseases in incapacitated individuals or those with weakened immune systems, therefore it is of importance to note that the bacterium which is known to be multi-drug resistant was sensitive towards the

leaf and root extracts of *B. natalensis* (**CVETANOVIC et al., 2019)**. The water extracts generally displayed weak bioactivity **RABE and VAN STADEN (1997)**, have reported that most water extracts exhibit very weak if not undetectable antibacterial activity in some plants. Petroleum ether is employed to extract fatty acids in plant material, this may be the reason for the potent antibacterial activity of the PE leaf extracts of the *B. natalensis*. A number of researchers **(RUSSELL, 1991; OUATTARA et al., 1997, WILLE and KYDONIEUS, 2003)** have demonstrated the antibacterial nature of fatty acids in plant extracts.

Numerous researchers **(CHENIA, 2013; FOMOGNE-FODJO et al., 2014; KONE et al., 2004; NAIDOO et al., 2013)**, have demonstrated a range of antibacterial effects of *K. africana* against different bacterial strains, bacteria were sensitive towards the leaf extracts of *K. africana* (DCM), *S. aureus, E. faecalis* and *E. coli* (0.31 and 0.02 mg/mL, respectively). No noteworthy activity against other bacterial strains (*E. faecalis* and *K. pnuemoniae*) were recognized. Moreover, the fruit extracts (ACE), exhibited bioactivity against (*S. aureus, E. faecalis* and *E. coli*), while water extracts only exhibited activity against (*S. aureus, E. faecalis*), however strong bacterial activities were displayed by ACE and DCM extracts. The stem bark and fruit of *K. africana* have been reported to show antibacterial activity **(McGAW et al., 2000; AKUNYILI et al., 1991)** to which the bioactivity may be attributed to the presence of iridoids **(AKUNYILI et al., 1991)**. Different plant parts displayed the presence of tannins (Chapter 2), these secondary metabolites have also been reported to have antibacterial activity **(VERMA et al., 2013)**. These findings concur with **HUSSAIN et al. (2016)**, who reported high sensitivity of *E. coli* toward ethanol leaf extracts of *K. africana* while *K. pneumonia* was resistant. Additionally, **SAINI et al. (2013)** reported maximum activity of the aqueous fruit extract against *S. aureus*. However, these results are in contrast with **McGAW et al. (2000)**, who reported antibacterial activity of the root and aerial parts of the plant tested against the *B. subtilis, E. coli, S. aureus* and *K. pnuemoniae* bacterial strains. Furthermore,

JEYACHANDRAN and MAHESH (2007), reported moderate antibacterial activity against *E. coli* and *S. aureus* from methanolic extracts of the plant and no activity from aqueous extracts. The difference in the acquired bioactivity of this plant may be due to geographical distribution, environmental stress as well as storage of the crude extracts **(RATES, 2001; STAFFORD et al., 2005)**. Different *K. africana* plant parts in this report have displayed strong inhibitory activity against the multi-drug resistant *K. pnuemoniae*, the bacterium responsible for causing infections in the skin, surgical wounds and urinary tract infections especially in diabetic patients **(NDHLALA et al., 2014).**

Leaf extracts of *L. leonurus* (PE) were active against *E. coli* (0.63 mg/mL), while DCM demonstrated activity towards *E. coli* and *E. faecalis*. The acetone extract was active against both Gram-positive and Gram-negative bacterial strains, but water extracts only exhibited weak antibacterial activity. The leaves of *L. leonurus* demonstrated the presence of some secondary metabolites which have been recognized as bioactive compounds (phenolics and flavonoids), exhibiting anti-inflammatory and antimicrobial activities among others **(OYEDEMI and AFOLAYAN, 2011; HUTCHINGS et al., 1996**). However, **KELMANSON et al. (2000)** reported no noteworthy antimicrobial activity of *L. leonurus* which is contrary to the results obtained in the present study. **JIMOH et al. (2010)**, reported that ACE and methanol extracts of the leaves of *L. leonurus* demonstrated antimicrobial activities (MIC 1-5 mg/mL) against a number of bacterial strains including *S. aureus* and E. *coli*, but that water extracts did not show antibacterial activity. *K. pnuemoniae* a bacterium frequently associated with nosocomial infections was susceptible to leaf extracts of *L. leonurus*, concurring with the obtained findings. The results are of significance considering the inhibition of *E. faecalis* by the leaf extracts as this bacterium has been identified as the most commonly isolated species in diabetic foot ulcers and is highly resistant to antibiotics such as erythromycin **(SHETTIGAR et al., 2018)**.

The PE and DCM extracts of stem bark of *S. birrea* extracts showed very weak bioactivity against the tested bacterial strains but the ACE and water extracts demonstrated strong inhibition of all bacterial strains. The results strongly concur with **ELOFF (2001)** who reported growth inhibition of the stem bark against the uropathogens (*S. aureus*, *Pseudomonas eruginosa*, *E. coli* and *E. faecalis*). **MOYO et al. (2011)** also reported the highest bioactivity against the Gram-positive and Gram-negative bacteria (MIC $\leq$ 0.195 and $<$ 1.0 mg/mL, respectively) from ethanolic *S. birrea* twig extracts. Similarly, **McGAW et al. (2000)** reported high antibacterial activity from mature *S. birrea* stem bark against *Bacillus subtilis* (0.0195 mg/mL) and *S. aureus* (0.049 mg/mL). The antibacterial activity of the *S. birrea* water extracts are in accordance with a number of studies that have reported good antibacterial activity of other plant water extracts i.e. *Alepidea natalensis* (**BUWA and VAN STADEN, 2006; MULAUDZI et al., 2009)**. The antibacterial activity of the *S. birrea* stem bark may be due to the presence of secondary metabolites reported to show antibacterial and anti-inflammatory activity **(CROZIER et al., 2006)**.

Gram-positive bacteria displayed more sensitivity to the plant extracts than the Gram-negative bacteria which demonstrated resistance against the extracts, owing to their complex cell wall which is comprised of a peptidoglycan-containing matrix and lipopolysaccharides which make it difficult for antibacterial agents to permeate through the cells **(MOYO et al, 2011)**.

Potent antifungal activity was demonstrated by a few plant extracts against *C. albicans*. The extracts of *K. africana* did not show any fungicidal activity. These results are in accordance with **HAMZA et al. (2006)** who reported that the fruit methanolic extracts of *K. africana* displayed activity only against *Cryptococcus neoformans* while other strains such as *C. ablicans* and *Colletotrichum papaya* were resistant. Although fatty acids have been reported to be potent antimicrobial agents **(WILLE and KYDONIEUS, 2003)**, the PE and DCM stem

bark extracts of *S. birrea* have shown no fungicidal activity. This is in agreement with **MASOKO et al. (2008)**, who reported inactivity of non-polar *S. birrea* solvent extracts against test organisms such as *Candida albidus* and *C. parapsilosis*. However, in the same study the acetone, ethanol and methanol extracts were found to inhibit the fungal organisms. Furthermore, **RUNYORO et al. (2006)**, reported the antifungal activity of the ethanolic extracts of the *S. birrea* stem bark against *C. ablicans*. The PE and ACE leaf extracts of *L. leonurus* displayed very strong antifungal activity (MIC 0.02 mg/mL), partly in agreement with **MAZIMBA (2015)**, who reported that the ethanolic and ethyl acetate extracts demonstrated moderate antifungal activities against of *C. albicans*, while the hexane and aqueous extracts did not show any activity. The DCM roots of *B. natalensis* also demonstrated very strong antifungal activity against the test organism. In line with these findings, **GHUMAN et al. (2016)**, reported potent antifungal activity from the chloroform leaf extracts of *B. natalensis* (0.63 mg/mL) against the test organism *C. albicans*. The different plant parts contain several polyphenols such as phenolics, flavonoids and tannins which have been reported to possess antifungal activity. This activity however, is partially dependent on the position and number of hydroxyl groups in the compounds and thus they may inhibit the microorganism either through a reaction with sulphur hydral groups or non-specific contact with proteins **(ARIF et al., 2009)**.

3.4 Conclusions

The plant extracts in this report have exhibited their abilities to inhibit both the diabetic related enzymes and antimicrobial organisms. Plant extracts exhibiting high α-glucosidase and lower α-amylase inhibitory activity are ideal for the treatment of DM and infections related to DM with possible minimal side effects. This enzyme activity combination prevents simultaneous inhibition of both enzymes, which could lead to abnormal bacterial fermentation of raw

carbohydrates in the gut causing side effects such as flatulence, and diarrhoea **(SEETALOO et al., 2019)**.

CHAPTER FOUR: EVALUATION OF MUTAGENICITY OF SELECTED PLANT EXTRACTS

4.1 Introduction

Medicinal plants have been utilized since time immemorial to treat numerous ailments including degenerative disorders such as diabetes mellitus (DM) **(SOFOWORA et al., 2013)**. These plants still form an integral part of primary health care in many developing countries including South Africa **(ELGORASHI et al., 2003; AREMU et al., 2013)**. Most individuals in developing countries prefer using medicinal plants over western or synthetic medicine **(GURUB-FAKIM, 2006)**. In South Africa particularly, about 60% of the population consult traditional healers with regards to either psychological, physical or metabolic disorders such as DM **(ELGORASHI et al., 2003)**. Presently there is a societal perception that natural products possess little to no toxicity while western medicinal products are believed to be toxic and cause undesirable side effects **(MATTANA et al., 2014)**. Often medicinal plants are consumed without any proof or knowledge about their safety **(MATTANA et al., 2014; VARGAS et al., 1991)**. The side effects of commonly used medicinal plants are not thoroughly recorded in the literature **(ELGORASHI et al., 2003)**, nonetheless, the World Health Organisation (WHO) has encouraged developing countries to supplement their health programs with complementary and alternative medicines (CAM), such as traditional medicine under the condition that they are non-toxic **(AKINTONWA et al., 2009; HONG and LYU, 2011)**.

Generally, plants are known to produce many secondary metabolites. These chemicals may be produced as a defence mechanism in response to environmental stimuli such as stress, infection or insect and herbivore attack **(CUZZOLIN et al., 2006)**. However, these secondary metabolites are vital sources of diverse chemical compounds with exceptional medicinal properties **(ARAUJO et al., 2015)**.

ARAUJO et al. (2015) defined genotoxicity as 'the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome' and according to **SHAH (2012)** this could eventually induce mutations in several body systems. As humans, we are continuously exposed to many chemical substances present in our environments which have the potential of being mutagenic **(BHATTACHARYA, 2011)**. These mutagens can be either chemical or physical agents capable of altering the genetic makeup and/or accelerate the occurrence of mutations **(MORTELMANS and ZEIGER, 2000)**. In living organisms, cellular system mutations may cause irreversible metabolic defects which may instigate morbidity and mortality **(BHATTACHARYA, 2011)**.

Numerous researchers have reported on the potential mutagenic and antimutagenic properties of some medicinal plants **(FERNANDES and VARGAS, 2003; VERSCHAEVE et al., 2004; VERSCHAEVE and VAN STADEN, 2008)**. Green plants are known to be a major source of antimutagens and organic toxic agents which may become cytotoxic and genotoxic if used over lengthy periods of time **(MATTANA et al., 2014; PLEWA and WAGNER, 1993)**. **ELGORASHI et al. (2003)**, further emphasised concern over potential mutagenicity posed by the long term use of medicinal plants since it has been discovered that certain plants which are used as food and medicine have had mutagenic effects through *in vitro* screening assays. Some chemical substances found in medicinal plants have been identified to be potentially toxic, thus presenting genotoxic activities **(DE SA´ FERREIRA and VARGAS, 1999)**. There is an increase in awareness of the poor quality and lack of scientific evidence with regards to the safety of plant products (**ARAUJO et al., 2015)**. A study by **DE SÁ FERREIRA and VARGAS (1999)** displayed possible toxicity and carcinogenic effects of a number of compounds found in some studied medicinal plants. A wide variety of natural mutagens and carcinogens are present in the human diet as a result of several environmental factors (i.e ultraviolet light or radon gas) **(VARGAS et al., 1991)**. Moreover, several workers have also

demonstrated the direct relationship between carcinogenicity and mutagenicity **(BARTSCH and TOMATIS, 1983; JONES and RICHARDSON, 1981)**. Therefore, as a means to ensure the safety of medicinal plants, it is important to screen the plants for their mutagenic potential **(VERSCHAEVE and VAN STADEN, 2008)**.

Due to their low cost, high specificity and speed, techniques involving *in vitro* trials which evaluate cytotoxicity and mutagenicity are being employed more frequently as alternatives to animal toxicity tests **(ASENSIO et al., 2007)**. A bacterial reverse mutation test requiring strains of either *Salmonella typhimurium* or *Escherichia coli* to detect point mutations of DNA is employed to screen for mutagenic activities in plant extracts **(ARAUJO et al., 2015)**. This test detects mutation through reverse mutations that exist in the test strains, thus restoring the performance of the bacteria to produce essential amino acids. Furthermore, the revertant bacteria is detected by their capability to grow without supplied amino acids **(OECD, 1997)**. The Ames test is a common and low cost bacterial mutagenicity test, widely employed to detect gene mutation by plant extracts using a common bacterial strain *S. typhimurium* **(MORTELMANS and ZEIGER, 2000; ARAUJO et al., 2015)**. This method involves reverse $His^- \rightarrow His^+$ mutations that can be observed through the plating of *S. typhimurium* grown in a histidine deprived growth medium **(MORTELMANS and ZEIGER, 2000; VERSCHAEVE and VAN STADEN, 2008)**. The use of different *S. typhimurium* bacterial strains allows for identification of the type of mutations occurring. For this reason, the present experiment was designed to screen the safety of some plant extracts demonstrating good antidiabetic inhibitory activities.

4.2 Materials and Methods

4.2.1 Preparation of plant extracts

Plant extracts were prepared as described in Chapter 2, section 2.2.2. The extracts were selected based on their complementary antidiabetic activity (high α-glucosidase and mild α-amylase inhibitory activity). The organic (ACE and DCM) extracts were dissolved with 50% DMSO to 10 mg/mL. The extract solutions were filter-sterilized using 0.22 μm Millipore filter tips. The filter-sterilized stock solutions were used to prepare working solutions (5000 μg/mL, 500 μg/mL, and 50 μg/mL) by simple dilutions with 50% DMSO.

4.2.2 Ames assay performed on selected plant extracts

The *S. typhimurium* microsome assay was used to evaluate mutagenicity of medicinal plant extracts that displayed good antidiabetic activities using tester strains TA102 and TA1535 without metabolic activation **(MARON and AMES, 1983; MORTELMANS and ZEIGER, 2000)**. The TA1535 strain, as a result of the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC), contains a hisG46 marker. Mutations that result in base pair substitution at the GC site can revert the hisG46 mutation to a wild type. The TA102 strain has an AT base pair at the hisG428 mutant site. Mutagens causing oxidative damage can revert hisG428 back to the wild type **(NDHLALA et al., 2010)**.

The different bacterial tester strains were inoculated and kept overnight in 10 mL Oxoid nutrient broth No. 2 for 16 h at 37 °C. The top agar supplemented with 0.5 mM histidine and biotin (100 mL of top agar and 10 mL biotin/histidine) was melted and kept in a 50 °C water bath throughout the preparation to prevent the agar from solidifying. One hundred microlitres of plant extract were dispensed into the allocated sterile tubes and further diluted with 500 μL

of phosphate buffer (adjusted to pH 7.4). To each test tube containing the extract mixture, the overnight bacterial cultures (100 μL) were added. Successively, 2 mL of enriched top agar was added to each tube and the resulting mixture was immediately mixed by vortexing and poured onto minimal plates and allowed to solidify and then incubated in an inverted position at 37 °C for 48 h. The tumorigenic chemical 4-nitroquinoline–N-oxide (4NQO) (2 μg/mL) was used as a positive control for the experiment whereas sterile distilled water was used as a negative control. After 48 h of incubation, the number of bacterial colonies were counted using a colony counter.

4.3 Results and Discussion

The Ames test was performed to determine and validate the potential genetic mutation that may be caused by the plant extracts, in the absence of the S9 metabolic activation which detects indirect mutations. The exogenous metabolic activation system (S9 mix) was unobtainable due to import challenges and so the indirect analysis of mutation by the plant extracts could not be carried out. The results of revertant colonies are presented in Table 4.1. **ZEIGER (2001)** explains that in order for a substance to be recognized as mutagenic it should produce a positive response with or without metabolic activation in any single bacterial strain.

Table 4. 1: The rate of mutagenic activity of selected plant extracts.

Plant species	Plant part	Solvent	Concentration (µg/mL)	TA strains	
				TA102	TA1535
Bulbine natalensis	Roots	DCM	5000	90.67±9.06	8.00±0.67
			500	114.00±6.51	8.67±2.08
			50	83.67±3.18	7.33±1.33
		ACE	5000	118.67±22.59	11.00±2.78
			500	187.67±9.84	7.00±1.00
			50	76.33±3.18	10.00±2.52
Kigelia africana	Fruit	ACE	5000	124.33±27.14	7.33±1.20
			500	101.00±6.00	8.00±1.15
			50	104.00±5.03	4.00±2.08
Sclerocarya birrea	Stem bark	ACE	5000	125.67±47.67	7.33±1.33
			500	116.67±5.36	7.00±1.53
			50	97.67±3.76	6.33±0.67
4NQO			2	237.00±25.77	210.67±15.53
Water			-	115.00±8.39	6.00±2.52

Number of revertant colonies: mean values of three triplicates. ACE= acetone, DCM= dichloromethane, 4NQO= 4-nitroquinoline–N-oxide. The assay was repeated two times.

According to **VERSCHAEVE and VAN STADEN (2008)** a plant extract may also be considered mutagenic if (a) there is an observed dose dependant increase in number of revertants, (b) the number of revertants is equal to or greater than two times the negative control. The organic plant extracts of *B. natalensis*, *K. africana* and *S. birrea* under investigation were not mutagenic against both the tester strains (TA102 and TA1535) without the S9 metabolic activation. The observed antimutagenic activity by these extracts means that the number of revertant colonies did not fulfil the criteria for mutagenicity. Extracts may be

classified as toxic, should the number of revertant colonies be drastically less than the negative control (spontaneous reversion) **(NDHLALA et al., 2010)**. In addition, no significant dose-dependent increase in the number of revertants by non-mutagenic extracts was observed. The non-mutagenic activities of the plant extracts may be due to organic compounds such as tannins and flavonoids which are reported to possess anti-mutagenic and anti-carcinogenic properties linked to oxidative activities that are involved in mutagenic deactivation **(HORN and VARGAS, 2003; KADA et al., 1985)**.

Non-mutagenic activity using the Ames test by most of the plant extracts against *S. typhimurium* as observed in the experiment is a positive step in establishing the partial safety of plant extracts. However, the negative response only confirms anti-mutagenicity towards the specific bacterial strains as well as their tested genetic endpoints and not their complete toxicology **(REID et al., 2006)**.

However, with regards to the stem bark extract (ACE) of *S. birrea*, the extract may be identified as a weak mutagen, as the number of revertant colonies is not two times the background number of colonies but demonstrated a slight dose dependant increase in the number of revertant colonies **(MORTELMANS and ZEIGER, 2000)**. Despite the significant pharmacology and phytochemistry of the stem bark of the *S. birrea* observed in this study, several authors withdrew their focus on the use of this plant as an antidiabetic traditional medicine **(OJEWOLE, 2003; VAN DE VENTER et al., 2008)**. This is largely due to reports by **OJEWOLE (2003)** stating that previously identified chemical compounds found in the bark extracts may be lethal to mammals. **VAN DE VENTER et al. (2008)**, who demonstrated toxicity effects of the plant extracts in cultured adipocytes and hepatocytes, further corroborated this finding. The extract of S. *birrea* stem bark has displayed high quantities of polyphenolic contents (Figure 2.2, 2.3 and 2.4). Though secondary metabolites including

flavonoids, terpenoids and tannins among others, have been reported to possess anti-mutagenic properties **(BHATTACHARYA, 2011)**, some mutagenic extracts have been linked to tannins and flavones as corroborated by **VARGAS et al. (1991)**. According to **MORTON (1980)**, high consumption of tannins and other anthocyanin's of plant material could lead to oesophageal cancer. Mutagenic agents may potentially cause fertility issues, generational mutations and induce cancer due to impairment of the germ line **(AREMU et al., 2013; MORTELMANS and ZEIGER, 2000)**.

4.4 Conclusions

It should be a priority to evaluate the potential risks of consuming medicinal plants before they can be utilised as remedies for various ailments. The S9 metabolic activation should also be performed to further establish the indirect mutagenesis of the plant extract.

CHAPTER FIVE: GENERAL CONCLUSIONS

The widespread scourge of non-communicable diseases like diabetes mellitus (DM) is a cause for concern. Despite the multitude of steps taken to improve the understanding and management of the disease and its related complications, DM still proves to be difficult to control and continues to affect millions of people worldwide, at an alarming rate. The World Health Organisation reported that diabetes is one of the major causes of morbidity and mortality of our era **(WHO, 2016)**. Synthetic medicines such as acarbose or insulin have been developed for the treatment of diabetes, however, a definite cure is yet to be discovered. Due to undesirable side effects (i.e. flatulence or abdominal pains) **(NASRI et al., 2015)**, caused by these synthetic medicines and often inaccessible health facilities, patients resort to the use of alternative therapies like indigenous medicinal plants and herbal medicines, which are believed to pose minimal side effects. Four traditional medicinal plants used in South Africa to treat diabetes were selected and subjected to biological screening to validate and assess their pharmacological efficacy, phytochemical composition and safety.

Antidiabetic inhibitory screening of plant extracts demonstrated a wide range of inhibitory activities towards the carbohydrate digesting enzymes (Figure 3.1 and 3.2). Moderate (30-49%) α-amylase inhibitory activity was displayed by extracts including the PE and ACE extracts of leaves of *K. africana*, ACE extracts of roots and PE leaf of *B. natalensis*, water extract of *L. leonurus* as well as DCM and ACE extracts of the stem bark of *S. birrea*. On the other hand, extracts exhibiting potent α-glucosidase activity consisted of the DCM and ACE extracts of roots of *B. natalensis*, ACE extracts of leaves of *B. natalensis* and ACE extracts of fruit of *K. africana* as well as ACE and water extracts of the *S. birrea* stem bark. These results demonstrate appreciable complementary inhibitory activities and they are considered desirable

and effective hypoglycaemic agents which could potentially preserve the integrity of pancreatic β-cells, thus reducing postprandial hyperglycemia.

DM compromises the immune system of patients and as a result they fall prey to pathogenic fungal and bacterial infections. Hence, the plant extracts were screened for antimicrobial (antibacterial and antifungal) activities. The extracts generally displayed good antimicrobial activity against test organisms, however, these extracts displayed more activity towards the Gram-positive strains. The DCM and ACE extracts of roots and the PE and DCM extracts of leaves of *B. natalensis*, the ACE and water extracts of fruit and the PE, DCM and ACE of the leaf extracts *of K. africana*, leaves of *L. leonurus* (ACE and DCM), and the ACE and water extracts of *S. birrea* stem bark demonstrated noteworthy antibacterial activity (MIC < 1 mg/mL) against some of the tested bacterial strains. The water extracts mostly displayed minimal antimicrobial activities (MIC > 1 mg/mL) but extracts of *S. birrea* (0.31 and 0.63 mg/mL) and fruit of *K. africana* (0.63 mg/mL) displayed the highest antibacterial activity. The plant extracts demonstrated maximum antifungal activity, but roots of *B. natalensis* (DCM) and leaves of *L. leonurus* (PE and ACE) and *S. birrea* (ACE and water) revealed high antifungal activity. Overall antimicrobial activities were observed more in organic plant extracts than water extracts, owing to the versatility of the solvents ability of extract varied compounds.

Oxidative stress plays a major role in the onset of diabetes and associated complications. Therefore, the plant extracts were subjected to antioxidant (β-Carotene; DPPH; H_2O_2 and FRAP) and phytochemical screening. The extracts generally demonstrated high scavenging abilities for free radicals. The ability of the plant extracts to delay β-Carotene bleaching (Table 2.2) was displayed by the ACE (86.49 %) extracts of roots of *B. natalensis*, ACE (94.58 %) and water (84.81 %) extracts of *S. birrea*, exhibiting ORR values lower than the reference drug BHT (0.18). The highest DPPH and hydrogen peroxide free radical scavenging activities (Table 2.3) were as a result of the ACE extract of fruit of *K. africana* (IC_{50} = 0.01 mg/mL)

which was superior to the reference drugs (ASC and BHT). The PE extracts of fruit of *K. africana*, DCM extracts of leaves of *L. leonurus* as well as the ACE and water extracts of the stem bark of *S. birrea* showed strong ferric reducing potentials. Secondary metabolites are reported to possess good pharmacological activities, forming a basis for the demonstrated biological activities of the plant extracts investigated. The preliminary screening (Table 2.4) of secondary metabolites of the different plant extracts displayed both the presence and absence of certain chemical compounds. All the methanolic extracts displayed the presence of flavonoids, carbohydrates and alkaloids. The extracts were further quantified for specific secondary compounds (total phenolic content, condensed tannins and flavonoids) which were demonstrated at different levels (Figures 2.2, 2.3 and 2.4). The *S. birrea* stem bark demonstrated very high polyphenolic contents as compared to the other extracts. Due to the groups of secondary metabolites identified, the plant species have demonstrated their potential to serve as natural antidiabetic medicines but also to serve as antimicrobial and antioxidant agents. These findings validate the traditional use of the selected plant species as antidiabetic agents and displayed their potentials to also prevent oxidative damage to cells.

The safety of consumed plant materials should be evaluated to ensure that the material is not toxic. The Ames test was performed to assess the mutagenic potential of the most active antidiabetic extracts. The extracts did not show mutagenic activity, but the ACE extract of *S. birrea* demonstrated weak mutagenic behaviour towards both the TA102 and TA1535 bacterial strains. The negative mutagenic response by some of the plant extracts requires further evaluation with other test strains (TA97, 98, 100, and 1537), including the S9 exogenous metabolic activation to establish accurate and conclusive results of their non-mutagenicity.

Recommendation

Findings of this research indicated that the stem bark of *S. birrea* had notable pharmacological properties, however the plant extracts (ACE) also demonstrated some weak mutagenic attributes suggesting that it is ill-advised for long term usage. This information should be incorporated into the indigenous knowledge systems (IKS) in order for locals to be aware of the health risks associated with the long term use of *S. birrea*.

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