

**EVALUATION OF THE MEDICINAL POTENTIALS OF *BULBINE*
ABYSSINICA A. RICH IN THE MANAGEMENT OF DIABETES
MELLITUS IN THE EASTERN CAPE, SOUTH AFRICA.**

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DECLARATION

I, Cromwell Mwiti Kibiti, declare that this thesis, submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Ethnobotany in the Faculty of Science and Agriculture, is my original work; and that this work has not been submitted at any other University for the award of any degree.

I also declare that I am fully aware of the University of Fort Hare policy on plagiarism and have taken every precaution to comply with the regulations of the University.

Again, I declare that I am fully aware of the University of Fort Hare policy on research ethics and was cleared to conduct my research.

Cromwell Mwiti Kibiti

Signature:  Date:

I confirm that the work reported here was carried out by the above named candidate under my supervision.

Prof Anthony Jide Afolayan

Signature: Date:

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GENERAL SUMMARY

Diabetes mellitus is a chronic physiological carbohydrate metabolic disorder with significant impact on the economy, quality of life and life expectancy in South Africa. Herbal medicine has become the alternative therapy in the management of this disease. However, their safety and effectiveness have not been investigated. To address this, one of the plants used in Eastern Cape Province, South Africa, *Bulbine abyssinica* A. Rich (Asphodelaceae), was evaluated.

Bulbine abyssinica is one of the species used in the management of diabetes mellitus. This plant was mentioned during an ethnobotanical survey conducted in Nkonkobe municipality of the Eastern Cape Province. Though a decoction prepared from the whole plant is used in the treatment of diabetes mellitus, the mechanism of action and its safety has not been elucidated. Thus, this research work was designed to contribute to the understanding of the possible mechanism of action of *B. abyssinica* as an antidiabetic medicinal plant and its toxic potentials to the users.

The aqueous extract exhibited remarkable inhibitory activity on α -amylase (estimated inhibitory concentration (IC)₅₀ value of 3.28 μ g/ml), while the acetone extract exhibited weak inhibitory activity. The acetone extract exhibited notable α -glucosidase inhibitory activity (IC₅₀ value of 4.27 μ g/ml) while aqueous extract had significantly weak activity. The Lineweaver-Burk double reciprocal plots revealed that the aqueous extract exerts non-competitive inhibition on the α -amylase activity while the acetone extract exerts a near competitive inhibitory pattern on the α -glucosidase activity.

The extracts from the plant possessed high free radical scavenging activities, with acetone extract exhibiting the highest activities in all assay models used except with ferric reducing power and nitric oxide (NO) scavenging ability. The aqueous extract exhibited the highest

ferric reducing power and nitric oxide radical mopping strength while the essential oil exhibited the highest scavenging activities with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and relatively high ferric reducing power and nitric oxide scavenging ability. The acetone extract and the essential oil of this species exhibited higher albumin denaturation inhibition than the aqueous extract while the latter showed the greatest membrane lysis protection.

The essential oil, acetone and aqueous extracts from this plant significantly inhibited the growth of *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*. *Klebsiella pneumoniae*, *Proteus vulgaris* and *Streptococcus pyogenes* growth were inhibited by acetone and aqueous extracts. The essential oil also showed inhibitory activity against *Proteus vulgaris*. However, the extracts were active against the growth of only three fungi species. The essential oil showed significant inhibitory activity against *Trichophyton rubrum*. The aqueous extract inhibited the growth of *Microsporum gypseum* while the acetone extract was active against *Microsporum canis*, and *Microsporum gypseum*.

The carbohydrate, crude fibre, moisture, ash, crude protein and crude fat of approximately 74.8%, 8.9%, 8.8%, 8%, 7.7% and 0.6%, respectively, were detected in this plant. The species is characterized by moderate levels of oxalates, phytic acids, Vitamin A, Vitamin C and Vitamin E. Potassium and calcium were present in highest levels, while magnesium, iron, sodium, aluminium and phosphorus were moderately present. Manganese, zinc and copper were in low amounts. These vitamins and mineral elements were within their recommended daily allowance (RDA) in humans. The investigation also revealed appreciable amounts of total phenols, flavonoids, flavanols, proanthocyanidins and alkaloids in both acetone and aqueous extracts while saponins and tannins were in trace amounts. The essential oil was characterized by large quantities of terpenes (91.9%) and small fraction of esters (8.01%). Amongst the identified phytochemicals, some have antidiabetic activities as

observed in this study. The large amounts of Terpinen-4-ol, γ -Terpinene, α -Terpinolene and α -Terpineol present in the volatile oil as the predominant compounds have been implicated in antioxidant, anti-inflammatory and antimicrobial activities suggesting their role in *B. abyssinica*'s therapeutic potentials.

Crystals composed of sodium, silicon, potassium and calcium were found to be the major constituents of the leaf, stem and the roots of this species when assessed using scanning electron microscope coupled to energy dispersive x-ray spectroscopy (EDXS). The X-ray analysis of the crystals in the leaf also showed the presence of iron and magnesium, while the stem had aluminium, phosphorous and magnesium. The roots also revealed the presence of sulphur and aluminium. The presence of these secretory products and their elemental composition suggest that crystals are the major secretory therapeutic products and are responsible for the observed metabolites and pharmacological properties of this plant. However, their secretory glands still remain unknown.

The essential oil, acetone and aqueous extract of this plant revealed relatively low toxicity on the hatching success of *Artemia salina* (brine shrimp) cysts with minimum inhibitory concentration (MIC) values of 2.90, 2.55, 3.26 mg/ml, respectively. Based on the Bastos's criterion of toxicity indices of the lethality test, all the plant fractions exhibited LD₅₀ values greater than 1 mg/ml, hence were considered non-toxic.

The present findings demonstrate that *B. abyssinica* acts as an antidiabetic agent possibly via altering carbohydrate metabolism, attenuating oxidative stress and opportunistic infections during diabetic status. These findings also suggest that this species is a rich source of these agents and a safe herbal remedy which could serve as a nutritional supplement in the diet which is essential in maintaining good health status. Further, these support the folkloric usage of this plant in the management of diabetes mellitus in the region.

CHAPTER ONE

GENERAL INTRODUCTION

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1.1 Background

1.1.1 Prevalence of diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder caused by abnormal metabolism of carbohydrate, promoted by factors namely, insulin deficiency and/or insulin resistance (Ozkol et al., 2013). The prevalence of diabetes globally was estimated to be 4.0% in 1995 and is projected to rise to 5.4% (300 million people) by the year 2025 (IDF, 2013). In 2010, 12.1 million people were estimated to be living with diabetes in Africa, and this is projected to increase to 23.9 million by 2030 (IDF, 2013). In South Africa, in 2011, the prevalence of diabetes was estimated to be 6.5% of the total national population. It is estimated to rise to 7.2% by the year 2030 (Whiting et al., 2011). Therefore, diabetes mellitus has affected several millions of people all over the world where it has a significant impact on the economy, health, quality of life and life expectancy of patients, as well as on the health care systems (Hall et al., 2011).

1.1.2 Types of diabetes mellitus

There are two major forms of diabetes mellitus: Type 1 diabetes mellitus previously known as Insulin dependent diabetes mellitus and Type 2 diabetes mellitus previously known as Non-insulin dependent diabetes mellitus (Bastaki, 2005). Type 1 diabetes is caused by failure to release insulin from the β -cells of the islets of langerhans in the pancreas. Type 2 diabetes is caused by insulin resistance probably due to too few insulin receptors. Other forms of diabetes include brittle diabetes and gestational diabetes (Bastaki, 2005).

1.1.3 Causes of diabetes mellitus

The major causes of Type 1 diabetes include genetic predisposition, environmental factors such as nutrition, exposure to viruses and allergens and autoimmunity leading to destruction of insulin secreting pancreatic β -cells (Patel et al., 2012). Autoimmunity is as a result of inflammatory mediators such as cytokines and free radicals (such as nitric oxide) generated by the cells leading to destruction of insulin producing pancreatic β -cells. The other major causes of Type 2 diabetes mellitus include genetic and environmental factors (Patel et al., 2012).

1.1.4 Complications of type I and type II diabetes mellitus

Type 1 diabetes requires insulin injection to prevent ketosis and other complications as well as maintenance of life (Patel et al., 2012). The complications of Type 1 and Type 2 diabetes mellitus include; retinopathy, neuropathy, angiopathy, nephropathy, infection and diabetic ketoacidosis (DKA) (Holt, 2004). Diabetic foot disease which is due to changes in blood vessels and nerves, often leads to ulceration and subsequent limb amputation. Skin disorders are also more common in diabetics. Infection is a major secondary diabetic complication. Diabetics are prone to develop bacterial (mycobacterium and anaerobic) and fungal infections (Casqueiro et al., 2012). This leads to ulceration and gangrene formation. These infections include; respiratory infections such as Streptococcus pneumonia, influenza, H1N1 and tuberculosis; urinary tract infections including asymptomatic bacteriuria, fungal cystitis, emphysematous cystitis, bacterial pyelonephritis, emphysematous cystitis and perinephric abscess. Gastrointestinal and liver infections are also common, and are caused by *H. pylori* infection, oral and oesophageal candidiasis, emphysematous cholecystitis, Hepatitis C, Hepatitis B and enteroviruses. Skin and soft tissue infections leading to foot infection,

necrotizing fasciitis and fournier's gangrene. Head and neck infections are as a result of invasive external otitis. Rhinocerebral mucormycosis are also common (Casqueiro et al., 2012).

1.1.5 Management of diabetes mellitus

The management of diabetes mellitus include diet therapy, mineral supplementation, physical activity, acupuncture and hydrotherapy (Pandey et al., 2011). The conventional management methods include; insulin therapy (exogenous insulin), oral glucose lowering agents such as sulfonylurea, biguanides, alpha-glucosidase inhibitors, thiazolidinediones and meglitinides (Holt, 2004). These conventional therapies are involved in stimulation of endogenous insulin secretion and enhancement of the action of insulin at the target tissues. The oral hypoglycemic agents (biguanides and sulfonylureas) inhibit the degradation of dietary starch by glycosidases such as α -amylase and α -glucosidase (Sudha et al., 2011). Inhibitors of pancreatic α -amylase delay carbohydrate digestion, causing a reduction in the rate of glucose absorption and lowering the post-prandial hyperglycemia (Sudha et al., 2011). Though these methods are used, they have varying side effects. The main side effects of these inhibitors are gastrointestinal disturbances including; bloating, abdominal discomfort, diarrhoea and flatulence (Sudha et al., 2011). Weight gain, atherogenesis, hypoglycaemia, skin reactions, insulin resistance due to antibody reaction, insulin lipodystrophy, visual disturbance and allergy are common side effects of insulin therapy (Pandey et al., 2011). Insulin therapy is also unavailable to many communities in developing countries due to cost and inaccessible health facilities (WHO, 2002).

Over the years, advancements in the management of diabetes have been made, including prevention of autoimmune attack and transplantation. There are several attempts made to

control autoimmune attack on the β -cells. In this, specific immuno-modulatory treatment is done while a substantial β -cells mass remains; that's during the prediabetic phase (Van Belle et al., 2011). For instance, the vitamin B-complex nicotinamide has been tried in Europe. Nicotinamide is postulated to protect against damage acting as an antioxidant and thus inhibits the deleterious effects of free radicals. It also inhibits the enzyme Poly (ADP-ribose) polymerase (PARP), thereby saving the cellular stores of Nicotinamide adenosine diphosphate (NAD). Furthermore, it stimulates islet cell proliferation (Sandler and Anderson, 1988). Another immunosuppressive compound that has been tried in newly diagnosed patients is cyclosporine A. This acts by inhibiting T-helper lymphocyte function (Sandler and Anderson, 1988). Unfortunately, cyclosporin A has potentially serious side effects, including a toxic action on the β -cell itself (Sandler and Anderson, 1988). Newer immunosuppressive drugs, such as FK-506 Transpl and Bacillus Calmette-Guerin (BCG) are under investigation though have great side effects (Wright et al., 1999).

Transplanting technology of either the pancreas or preparations of islet tissues has also been tried. However, it is limited by the problem of obtaining donor tissue and preventing immune rejection of the graft (Qi, 2014). Nevertheless, transplantation is yet the only available treatment that can lead to insulin independence. Human allograft transplantation cannot be used on a large scale in clinical practice. After whole pancreas transplantation, the graft survival after one year is 85-90%. Islet transplants are much more vulnerable. Many of them fail within few weeks or months after engraftment (Qi, 2014). The reasons for these functional failures are largely unknown, although insufficient numbers of islets, engraftment difficulties, chronic rejection and recurrence of autoimmune disease have been suggested to

be contributing factors. Moreover, hyperglycaemia in the recipient after transplantation deteriorates islet graft survival and function (Jung et al., 2009).

One of the major obstacles for clinical islet transplantation is lack of donors. Therefore, it is important to optimize the number of β -cells harvested from each donor, stimulate the growth and/or differentiation of β -cells or to genetically manipulate insulin producing cell lines for transplantation (Qi, 2014). The differentiated β -cells have the ability to proliferate at a low pace. The proliferation rate can be affected in many ways, for example, by growth stimulating hormones like growth hormone and prolactin. Also the size and composition of the graft and the blood glucose level in the recipient are of crucial importance for β -cell replication (Jung et al., 2009; Qi, 2014). Therefore, though the conventional therapy is effective, there are numerous drawbacks in their use. This has led to seeking alternative therapy in herbal medicine.

1.2 Herbal medicine

Medicinal plants have been used in traditional medicines, since ancient times to maintain health or cure. These plants contribute about 50% of the total drugs used in the world today (Pan et al., 2013). Over many decades, there has been an increase in the use of herbal medicine in primary health care because of their wide biological activities, lower side effects and accessibility (Ekor, 2013). Herbal medicines continue to play key role in diabetic therapy, particularly in the developing countries where most people have limited resources and inaccessibility to conventional therapies (Ekor, 2013). In South Africa, about 38 out of the 3,000 indigenous medicinal plant species have been authenticated as phytomedicines for ailments including diabetes mellitus (Van Wyk, 2008).

Before and after the introduction of insulin in the treatment of diabetes mellitus in 1922, dietary and medicinal plant remedies had been commonly used. The increase in demand for the use of plant based medicines to treat diabetes may be due to the side effects associated with the use of orthodox drugs (insulin and oral hypoglycemic agents) and the belief that herbs do provide some benefits over and above allopathic medicine and allow the users to feel that they have some control in their choice of medication (Afolayan and Sunmonu, 2010). Moreover, the use of herbal medicine for treatment of diabetes has been legitimized by World Health Organization (WHO) (Afolayan and Sunmonu, 2010). This has led to advancements of phytomedicine facets including ethnobotany, biochemistry, molecular biology, computational biology and information technology. This has enhanced the understanding of the mechanism of action of many herbal drugs in relation to the existing synthetic drugs (Pan et al., 2013). Antidiabetic medicinal products are now generally available in both developed and developing countries (Pan et al., 2013).

1.2.1 Mechanism of action of antidiabetic plants

Plant remedies have been a rich source of new anti-diabetic drug discovery. These antidiabetic properties have been widely attributed to the presence of metabolites which include fibres, vitamins, macro and micro elements, phytochemicals and other nutrients (Atangwho et al., 2009). For example, metformin the most commonly used antidiabetic drug was discovered from *Galega officinalis* whose activity is due to richness in guanidine (Pulito et al., 2013). The ginseng species; *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (American ginseng) possess antidiabetic property attributed to ginsenosides (steroidal saponins) (Qi et al., 2011). Onion (*Allium cepa*) and garlic (*Allium sativum*) contain bioactive sulfur-containing compounds, allyl propyl disulfide (APDS) and diallyl disulfide (allicin),

respectively. These active ingredients lower glucose levels by competing with insulin (which is also a disulfide) for insulin-inactivating sites in the liver, resulting in an increase of free insulin (Marles and Farnsworth, 1995). *Trigonella foenum graecum* which contains the alkaloid trigonelline, nicotinic acid and coumarin have been shown to lower the glucose and lipid lowering effects in diabetic patients. The fiber constitutes potential mechanisms of fenugreek's beneficial effect in diabetic patients (Ngugi et al., 2012). Bitter gourd (*Momordica charantia*) has been extensively used in folk medicine as a remedy for diabetes. The active, hypoglycemic constituents include charantin, obtained from an alcohol extract of the fruit, and a polypeptide called p-insulin (plant insulin or polypeptide-p) isolated from the fruit and seeds of the plant. Although the precise mechanism of action remains to be fully elucidated, the p-insulin is postulated to stimulate insulin release or possibly glycogen synthesis in the liver (Wang et al., 2013).

South African medicinal plants that have been investigated include *Aloe arborescens*, *Brachylaena discolor*, *Cissampelos capensis*, *Clausena anisata*, *Ficus lutea*, *Harpagophytum procumbens*, *Hypoxis hemerocallidea*, *Leonotis leonurus*, *Momordia foetida*, *Nelumbo nucifera*, *Psidium guajava* and *Vernonia amygdalina* (Van de Venter et al., 2008). Their mechanism of action in diabetic status alleviation involves inhibiting glucokinase and glucose 6 phosphatase activities, stimulating glucose utilization in adipocytes, acting as α -amylase inhibitor in the gut, improving glucose uptake into the cells, increasing glucose utilization in the liver and ameliorating oxidative stress (Van de Venter et al., 2008).

There are many other classical examples of these antidiabetic plants whose mechanisms of action have been experimentally demonstrated in *in-vivo* and *in-vitro* diabetic models and are well documented. The elucidated mechanisms of action of these antidiabetic plants are

outlined in Figure 1. These include inhibition of mitochondrial function, stimulation of glycolysis, activation of AMPK (adenosine mono-phosphate kinase) pathway, suppression of adipogenesis, uptake of glucose and induction of low density lipoprotein (Edwin et al., 2008). Some plants with anti-diabetic properties inhibit carbohydrate digestive enzymes such as α -glucosidase and α -amylase (Cummings et al., 2004; Kibiti and Afolayan, 2015). Ameliorating oxidative stress and modification of insulin structure or insulin receptor sensitivity as well as up-regulation of glucose transporter by some plants have been reported in several studies (Tanira, 1994)

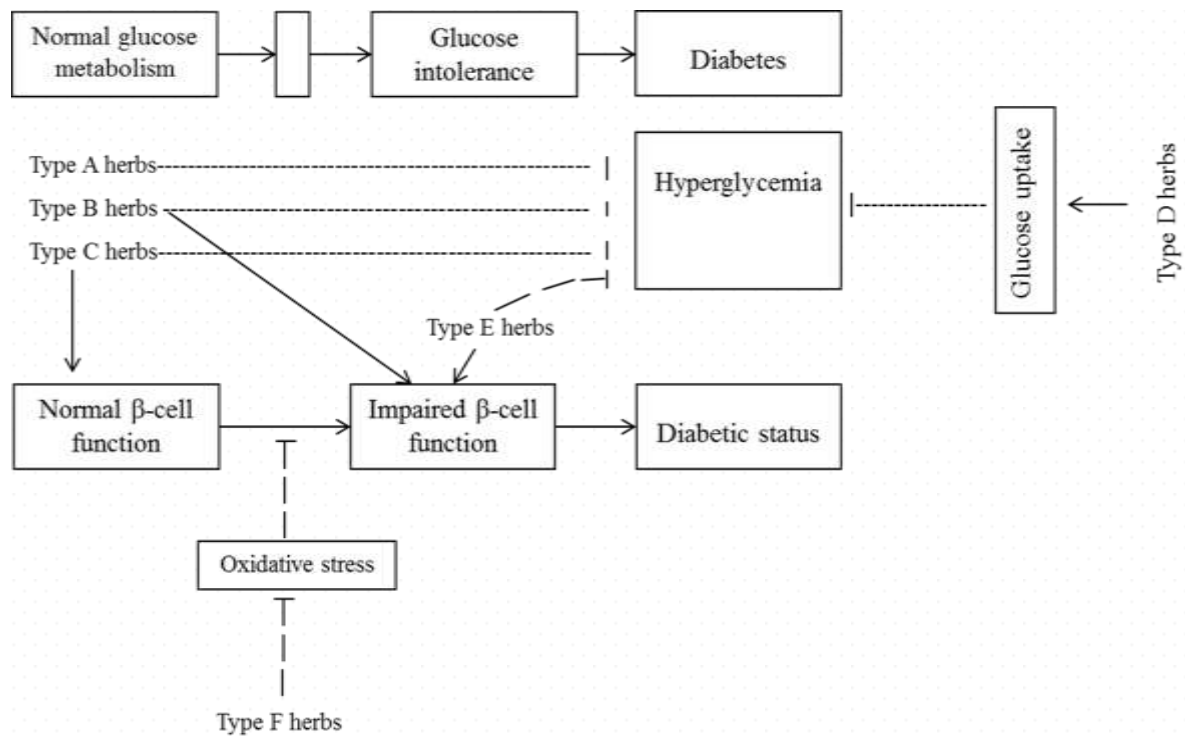


Figure 1: Mechanisms underlying plants used in traditional medicine in the management of diabetes mellitus. Some inhibit intestinal absorption of glucose and alter glucose metabolizing enzymes (type A herbs); have insulin mimetic properties (type B herbs); potentiates glucose-induced insulin release (type C herbs); enhances peripheral glucose uptake (type D herbs); promotes regeneration of β -cell of islets of Langerhans (type E herbs); and ameliorates oxidative stress (type F herbs).

1.3. The choice of *Bulbine abyssinica* for this study

At the beginning of this study, *B. abyssinica* A. Rich (Asphodelaceae) was chosen for this study based on an ethnobotanical survey done by Oyedemi et al. (2009). The survey showed that the whole plant parts of this plant are crushed, boiled and the decoction is used by traditional healers in the management of diabetes mellitus in Nkonkobe municipality, Eastern Cape Province of South Africa (Oyedemi et al., 2009).

1.3.1 The description of *Bulbine abyssinica*

Bulbine abyssinica is known as Bushy bulbine (English), intelezi (Xhosa), ibhucu (Zulu), moetsa-mollo (Sotho) and geelkatstert, wildekopieva (Afrikaans) (Pooley, 1998). This species is a succulent perennial herb with rhizomatous base which grows in small clusters. The plant is a water-wise plant with flowers and fruits having yellow and black colours (Pooley, 1998). The roots are many, slender or swollen. It has soft, dark green grass-like leaves which grow up to 35 cm long. Mature fruits are black, 4 mm in diameter and often covered with the faded perianth persisting as a cap. *B. abyssinica* occurs from the Eastern Cape, through KwaZulu-Natal, Swaziland, Lesotho and further north to Ethiopia. This plant grows in all weather and favours rocky grassland and shallow soil overlying rock (Pooley, 1998). It can also grow in woodlands and along seepage areas (Figure 2).



Figure 2: *Bulbine abyssinica* A. Rich (A) growing in its natural habitat in lower Ncera location in Nkonkobe Municipality of the Eastern Cape Province, South Africa, (B) whole plant parts of the plant collected for study.

1.3.2 Traditional uses

In South Africa folk medicine, *B. abyssinica* is used in the treatment of rheumatism, dysentery, bilharzia and cracked lips (Van Wyk et al., 2008). The root decoction is used in the treatment of infertility and back pain. The tea leaf is used to treat cough, vaginal and bladder infections (Van Wyk et al., 2008). Currently, a decoction is prepared from the whole plant by the traditional healers for the management of diabetes mellitus (Oyedemi et al., 2009).

1.3.3 Phytochemical composition

Several phytochemicals have been isolated from *B. abyssinica*. For example, some anthraquinones have been isolated from the roots (Bezabih et al., 1997). From the fruits, three new dimeric anthracene derivatives namely; abyquinone A, abyquinone B and abyquinone C have also been isolated. Anthraquinones, phenylanthraquinones and isofuranonaphthoquinones have been isolated from the roots, leaves and fruits of this species (Bringmann et al., 2002). The phenylanthraquinone, bulbine-knipholone has been isolated from the roots. This compound showed *in-vitro* antiplasmodial activity and no cytotoxic effects on mammalian cells (Bringmann et al., 2002).

Despite the advances achieved to scientifically validate some of *B. abyssinica*'s chemical structures and bioactivities, and the acclaimed folkloric use of this plant as an antidiabetic agent and in treatment of other diseases, there is dearth of scientific evidence to substantiate these claims. Therefore, this study aimed at providing information on the antidiabetic properties, probable mechanisms of action and toxicity effect of this plant with a view to validating its acclaimed use in Eastern Cape Province folk medicine. At the beginning of this study, there was no information in literature on the above stated properties of *B. abyssinica*.

1.4 Rationale and justification for this study

The current prevalence of diabetes mellitus worldwide constitutes a global public health burden (Olokoba et al., 2012). Despite the development of new drugs to manage the disease, it is predicted to hit 300 million people by 2025. In 2011, South Africa's 6.5% of the population had the disease, and it was projected to rise to 7.2% by 2030 (IDF, 2013). The prevalence of diabetes mellitus has impaired the economy and quality of life in South Africa. This is partially contributed by the inefficiency in management of this disease using

conventional therapy such as insulin or oral hypoglycaemic drugs that normalize blood glucose homeostasis (Balamurugan et al., 2014). Moreover, these drugs have side effects such as hypoglycemia, weight gain, edema, abnormal liver function and diarrhea (Tabatabaei-Malazy et al., 2015). They are also very expensive and as a result they are inaccessible to the patients living in the rural areas. Owing to this fact, diabetic individuals have resorted to the use of herbal medicines with antidiabetic potential and probable fewer side effects (Khan et al., 2012). Some of these antidiabetic plant remedies have been scientifically authenticated but a large number of them remain unexplored. Further studies on pharmacological, mechanisms of actions and toxicity properties of these plants are necessitated to justify their folkloric usage (Ashafa, 2013).

Therefore, in order to authenticate one of the antidiabetic plants used in Eastern Cape Province, *B. abyssinica* was selected based on a previously ethnobotanical survey. Selected pharmacological, chemical composition and toxicity analysis were done to validate its use and safety to guarantee users in the folk medicine.

1.5 Objectives of the study

The overall objective of this study was to investigate the antidiabetic property of *B. abyssinica* by evaluating possible mechanism of action and their toxicity potentials. This is with the view of validating the folkloric use of this plant in the management of diabetes mellitus in the Eastern Cape Province, South Africa.

The specific objectives are:

1. To investigate the *in vitro* α -amylase and α -glucosidase inhibitory activities of the aqueous and acetone whole plant extracts of *B. abyssinica*.

2. To evaluate the *in vitro* antioxidant and anti-inflammatory activities of the essential oil, aqueous and acetone extracts of the plant.
3. To investigate the *in vitro* antibacterial and antifungal activities of the plant fractions against a variety of pathogenic bacteria and fungi implicated in secondary infections associated with diabetes mellitus.
4. To identify the nutritive and antinutrient value, elemental composition, phytochemical constituents and compounds responsible for the pharmacological properties of this plant.
5. To examine the ultrastructure and morphology of the leaf, stem and roots of this plant using the scanning electron microscope.
6. To determine the toxicity potential of the plant fractions using brine shrimp test.

1.6 Description of the study area

The whole plant parts of *B. abyssinica* including the leaves, flowers, stems and roots were collected from lower Ncera location in Nkonkobe Municipality of the Eastern Cape Province. This area is located at latitudes 30° 00' to 34°15'S and longitudes 22° 45' to 30° 15'E. It is bounded by the sea in the East and the drier Karoo (semi-desert vegetation) in the West. The elevation ranges from sea-level to approximately 2,200 m in the North of the Province (Afolayan and Wintola, 2014). The major ethnic group is Xhosa speaking people with farming as their main occupation. The majority of people in this region are rural dwellers, hence rely on herbal medicine in addition to conventional therapies in treatment of diseases, including diabetes mellitus (Oyedemi et al., 2009).

1.7 The structure of this thesis

This thesis is composed of chapters that have been published, accepted or under review in various peer-reviewed and accredited journals. The general introduction is in Chapter 1. Chapter 2 is composed of the scope of the study. Chapter 3 presents the results of *in vitro* α -amylase and α -glucosidase inhibitory activities of the acetone and aqueous whole plant extracts of *B. abyssinica*. Chapter 4 is composed of the evaluation of the antioxidant and anti-inflammatory activities of the essential oils, aqueous and acetone extracts of the plant. The investigations of the *in vitro* antibacterial and antifungal activities of these plant fractions against a variety of selected opportunistic isolates causing secondary infections associated with diabetes mellitus are reported in Chapter 5. Chapter 6 is composed of the report of the nutritive value, elemental composition, phytochemical constituents and compounds present in this plant. The identity and the known pharmacological properties from literature for these compounds in relation to the bioactivities in this study are also discussed therein. Chapter 7 presents the results of the micromorphology of the plant's parts and the probable storage sites of these bioactive compounds. In chapter 8, the profiling of toxicity potential of the plant species using brine shrimp test is presented. The general discussion, conclusions and contribution to knowledge emanating from the entire study are presented in Chapter 9.

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CHAPTER 2

SCOPE OF THE STUDY

CHAPTER TWO

Scope of the study

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Scope of the study

In order to evaluate the possible mechanism of action and the toxicity potential of *B. abyssinica*, its essential oil, acetone and water (aqueous) whole plant extracts were used to carry out several *in vitro* experiments and their chemical composition analyses done using standard procedures.

The essential oil and water extractions were selected on the basis that the traditional practitioners prepare this herbal medicine as a decoction (Oyedemi et al., 2009). Acetone was selected as an extractant because it dissolves both hydrophilic and lipophilic components from plants (Olaokun et al., 2013). Acetone is also volatile, and has low toxicity for use in microbial bioassays (Olaokun et al., 2013). Besides, acetone does not extract sugars which if present (as would be the case with water and alcoholic extracts) would complicate gastrointestinal enzymatic inhibitory assays such as α -amylase and α -glucosidase (Olaokun et al., 2013).

1.1 The *in vitro* activity of *B. abyssinica* extracts on enzyme inhibitory activities

Currently, one therapeutic approach for treating diabetes mellitus is to decrease the post-prandial hyperglycaemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase in the gastrointestinal tract. Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently lessening the post-prandial plasma glucose rise (Ali et al., 2006). New anti-diabetic compounds which function by this mechanism but are devoid of side effect are therefore desirable (Deepa et al., 2013). This was the first time that the whole

plant extracts of *B. abyssinica* were evaluated for their antidiabetic property using *in vitro* α -amylase and α -glucosidase assays.

1.2 The *in vitro* antioxidant and anti-inflammatory activity of the plant

Oxidative stress results from imbalance between the formation and neutralization of free radicals (Rangasamy and Namasivayam, 2014). Oxidative stress initiated by reactive oxygen species (ROS) damages cellular macromolecules such as DNA, proteins and lipids (Rangasamy and Namasivayam, 2014). Among the effects, lipid peroxidation initiates inflammation processes, indicating the latter is intertwined to oxidative stress. Thus, free radicals are vital mediators that provoke/sustain inflammatory processes and consequently, their neutralization by antioxidants can attenuate inflammation (Murugan and Parimelazhagan, 2014). These cellular oxidative damage results in diseases including diabetes mellitus, atherosclerosis, myocardial infarction among others (Murugan and Parimelazhagan, 2014).

Excessive generation of free radicals like nitric oxide initiates β -cell destruction leading to autoimmune destruction. This triggers inflammatory cytokines targeting the pancreatic islets, leading to pathogenesis of inflammation and ultimately causing type 1 diabetes mellitus (Keane et al., 2015). Similarly, these radicals have also been implicated in type 2 diabetes in activating the β -apoptotic pathways, impairing insulin synthesis and thus contribute to insulin resistance (Tangvarasittichai, 2015). Thus, antioxidants are reducing agents that can limit cellular oxidative stress hence attenuating inflammation and serving as antidiabetic agents (Umapathy et al., 2010).

Plant remedies are natural antioxidants due to the presence of secondary metabolites which act as antioxidant agents. Over the years, the mechanism of action of some antidiabetic plants have been shown to be via free radical scavenging activity of free radicals generated at the onset and during diabetic state in the experimental animals (Rice-Evans et al., 1995). Therefore, the use of herbal remedies as natural antioxidant and anti-inflammatory agents with no side effects is postulated to be a milestone for the cure of oxidative stress generated diseases such as diabetes mellitus. At the beginning of this study, there was no literature on the antioxidant and anti-inflammatory activities of *B. abyssinica*. Therefore, one of the objectives of this study was to evaluate the whole plant part of *B. abyssinica* for its antioxidant and anti-inflammatory activities.

1.3 Antibacterial and antifungal properties of the plant

Free radical scavenging deficiency ascribes to inflammation of cells and tissues. Microbial invasion during diabetes mellitus status and in other disease conditions is attributed to the host having susceptible damaged cells due to inflammation (Skarbez et al., 2010). Diabetic patients are prone to develop bacterial and fungal infections as secondary complications during this stage. Some of these infections cause diabetic foot diseases, gastrointestinal, pulmonary and respiratory failure, limb amputation and even death (Holt, 2004). To attenuate this diabetic status, there is need to treat these infections. These infections are treated using the conventional antibiotics. However, the treatment using plant remedies has been on the rise in African traditional medicine (Mishra et al., 2007; Mahesh and Satish, 2008). Despite this species being used in South Africa folk medicine in the treatment of infections, there is no scientific data to validate it. Therefore, development of new antidiabetic drugs with multi-pharmacological properties including treatment of secondary infections associated with

diabetes mellitus, attenuating inflammation and oxidative stress could be desirable. At the beginning of this study, there was no literature available of antibacterial and antifungal properties of *B. abyssinica*.

1.4 Holistic nature of the plant and identification of bioactive metabolites

Medicinal plants are sources of bioactive compounds which are crucial in the development of novel drugs (Cragg and Newman, 2013). The use of conventional drugs which are expensive, inaccessible and with side effects makes it imperative to use plant remedies which are cheap, fast acting and readily available to the rural community. It thus, becomes vital to study the holistic nature of the herbal medicine and identify the bioactive metabolite (s) in the whole plant extract of *B. abyssinica*. This information would partially justify the traditional use of the plant by the healers and would further enhance the confidence of the healers in their ethno-medicinal practice. Therefore, one of the objectives of this study was to show the nutritive value, phytochemical constituents and identification of active metabolites using GC-MS analysis. Further, characterization and confirmation of their pharmacological properties was done using empirical searches in the available databases.

1.5 Identification of the secretory sites of the bioactive compounds/metabolites

Ultra-structural and morphological studies of plant secretory organs have played crucial role in bioprospecting and drug development. These studies shed light on the nature of the secretory organs/sites and the secreted material with their possible functional therapeutic significance (Afolayan and Meyer, 1995). Production of bioactive compounds with their therapeutic properties has been reported in many plant species. In most cases, the source of these bioactive compounds has been attributed to the glandular trichomes (Afolayan and Meyer, 1995). Crystals known to play key roles in plant's defense system have also been

reported to have great therapeutic values including healing cuts, wounds and also in fractures (Masram and Harisha, 2012).

Therefore, in this study, one of the objectives was to evaluate the ultra-structural morphology and elemental composition of the leaves, stems and roots of *B. abyssinica* using scanning electron microscope (SEM) coupled to Energy dispersive X-ray spectroscopy (EDXS) with the aim to identify the possible secretory sites and their products and to relate the findings to their possible functional role in the production of therapeutic compounds.

1.6 Toxicity assessment of *B. abyssinica* using Brine shrimp test

Plant remedies consist of biologically active compounds such as cyanogenic glycosides, glycoalkaloid, oxalic acid derivatives and alkaloids, among others. Some of these compounds are highly toxic when ingested at certain concentrations (Oyedemi et al., 2010). There are several reports of adverse side effects alleged from the use of medicinal plants in African folk medicine (Street and Prinsloo, 2013). These could be attributed to the presence of phytotoxic compounds in the plant extracts and lack of knowledge of actual dosage necessary for the treatment of diseases (Oyedemi et al., 2010). Therefore as the use of these plants increases, there is need to screen for toxicity to assure safety to the users (Ouedraogo et al., 2012).

The invertebrate, *Artemia salina* Leach (brine shrimp) test is widely used for preliminary evaluation of the general toxicity of herbal remedies. Then, other *in vivo* and *in vitro* cytotoxicity assays are used to validate these toxicity activities (Rajabi et al., 2015). Therefore, an attempt was made in this present study, one of its first kind, for scientific investigation on the toxicity of whole plant part of *B. abyssinica* using the *A. salina* hatching

and lethality assays. The long term objective of this research is to provide a baseline for future research in the development of novel safe antidiabetic drugs from this medicinal plant.

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CHAPTER 3

***IN VITRO* ANTIDIABETIC PROPERTIES OF *BULBINE ABYSSINICA*.**

CHAPTER THREE

In vitro antidiabetic properties of *B. abyssinica*.

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Introduction

Pancreatic α -amylase is a key enzyme in the gastrointestinal tract and catalysis the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose and maltotriose (Mohamed et al., 2012). Subsequently, these are then acted upon by α -glucosidases and further degraded to glucose which on absorption enters the blood-stream leading to elevated blood glucose levels (post-prandial hyperglycemia). Studies have shown that there is a positive correlation in activity of human pancreatic α -amylase in the small intestine with an increase in post-prandial glucose levels. Therefore, this has formed a rational in the treatment of diabetes mellitus and development of novel antidiabetic drugs (Mohamed et al., 2012).

Currently, the available conventional therapies for diabetes mellitus include synthetic insulin and oral hypoglycemic agents, such as biguanides and sulfonylureas (Halimi et al., 2008). These oral agents act through inhibition of degradation of dietary starch by glycosidases such as α -amylase and α -glucosidase (Coman et al., 2012). Some of the synthetic inhibitors currently being clinically used include acarbose and miglitol which inhibit glycosidases such as α -glucosidase and α -amylase while others such as voglibose inhibit α -glucosidase (Derosa and Maffioli, 2012). Though these synthetic medications are effective, they have drawbacks which include cost and serious side effects (Mallare et al., 2005). This has led to seeking alternatives in plant remedies since they are available and less expensive when compared to synthetic hypoglycemic agents (Mallare et al., 2005). Moreover, novel drugs with this type of mechanism of action with no side effects are more desirable (Deepa et al., 2013).

Medicinal plants possess antidiabetic properties due to a wide range of phytochemical compositions (Joseph and Jini, 2013). Moreover, the majority of plants used in South Africa folk medicine need to be thoroughly explored to understand the mechanism of action underlying their activity. *B. abyssinica* is among the species used in South Africa as a folk remedy for the management of this disease (Oyedemi et al., 2009). However, there is no scientific evidence available in literature to support its antidiabetic activity.

The present study was therefore designed to investigate the antidiabetic properties of the plant using *in vitro* enzyme models (α -amylase and α -glucosidase inhibitory assays) designed to simulate specific known antidiabetic targets.

Materials and Methods

Chemicals

The α -amylase (1, 4- α -D-Glucan glucanohydrolase; EC 3.2.1.1) from *Aspergillus oryzae*, α -glucosidase from *Saccharomyces cerevisiae* and para-nitrophenyl α -D-glucopyranoside, soluble starch, acarbose and 3, 5-Dinitrosalicylic acid were obtained from Sigma-Adrich Co, St Louis, USA. Other chemicals and reagents used were of analytical grade and distilled water was used.

Plant materials

The whole plant parts of *B. abyssinica* including the leaves, flowers, stems and roots were collected from lower Ncera location in Nkonkobe Municipality of the Eastern Cape Province. The plant samples were then authenticated and the voucher specimen (KibMed 2014/01) was deposited in the Giffen's Herbarium, University of Fort Hare, South Africa.

Preparation of plant extracts

The plant samples were air-dried, ground to homogeneous powder and extracted using acetone and water. For acetone extraction, the solvent and ground samples were mixed on a shaker (Orbital Incubator Shaker, Gallenkamp) at 140 rev/min) for 48 h, while aqueous extraction was done by boiling the samples in distilled water for 30 min and allowed to cool. These extracts were then filtered using a Buchner funnel and Whatman No. 1 filter paper. The filtrate obtained with water extraction was frozen at -40°C and freeze dried for 48 h using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY, USA). The acetone extracts were concentrated to dryness under reduced pressure at 57°C using a rotary evaporator (Strike 202 Steroglass, Italy). The resulting extracts were reconstituted with their respective solvents to give the desired concentrations used in the study. The yield was 8.3 and 5.8 g for acetone and aqueous extracts, respectively. The dried crude extract was dissolved in 10% dimethylsulphoxide (DMSO) to yield a stock solution from which lower concentrations were prepared, according to the method described by Kazeem et al. (2013).

Preliminary screening of *B. abyssinica* for enzyme inhibitory activities

Alpha-amylase inhibitory activity

The effect of crude *B. abyssinica* extracts on α -amylase activity was determined using a microtitre plate according to the method described by Xiao et al. (2006) and Kazeem et al. (2013) with some modification. Briefly 0.5 μ l of α -amylase (0.5 mg/ml) was pre-incubated with 12.5 μ l of acetone and aqueous extracts of *B. abyssinica* (5 mg/ml to 2.44 μ g/ml) for 15 min at 37°C in a water bath. The reaction was started by addition of 12.5 μ l of 1% starch dissolved in 0.02 M sodium phosphate buffer (pH 6.9) after every 5 sec, 5 times. The reaction mixture was then incubated at 37°C for 20 minutes and terminated by addition of 25

µl of DNS reagent (96 mM 3, 5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH). The reaction mixture was heated for 15 min at 100°C and allowed to cool at room temperature. The reaction mixture was then diluted by adding 200 µl of water. The absorbance was measured at 540 nm in an automatic ELISA microplate reader (SynergyMx BiotekR, USA). A control was prepared using the same procedure except that the plant extract was replaced with distilled water. The α-amylase inhibitory activity was calculated as;

$$\% \text{ Inhibition} = \{(Ac - Ae)/Ac\} 100;$$

Where; Ac and Ae are the absorbance of the control and extract, respectively.

Alpha glucosidase inhibitory activity

The effect of the plant extracts on α-glucosidase activity was determined using a microtitre plate according to the chromogenic method described by Xiao et al. (2006) and Kazeem et al. (2013) with some modification, using α-glucosidase from *Saccharomyces cerevisiae*. The substrate solution 5 mM p-nitrophenyl glucopyranoside (pNPG) was prepared in 0.1 M phosphate buffer, pH 6.9. A 50 µl (1.0 Unit/ml) of α-glucosidase were pre-incubated with 25 µl of acetone and aqueous extracts of *B. abyssinica* (5 mg/ml to 2.44 µg/ml) for 30 minutes at 37°C. A 25 µl of pNPG was then added to start the reaction. The reaction mixture was incubated at 37°C for 30 minutes and stopped by adding 30 µl of 0.1 M Na₂CO₃. The α-glucosidase activity was determined by measuring the yellow colored p-nitrophenol released from pNPG at 400 nm in an automatic ELISA microplate reader (SynergyMx BiotekR, USA). The α-glucosidase inhibitory activity was calculated as follows;

$$\% \text{ Inhibition} = \{(Ac - Ae)/Ac\} 100;$$

Where; Ac and Ae are the absorbance of the control and extract, respectively.

Enzyme inhibitor effectiveness of *B. abyssinica* extracts

The effectiveness of *B. abyssinica* extracts showing strong enzyme inhibitory activities was evaluated in terms of their IC₅₀ values and compared with that of known synthetic enzyme inhibitor (acarbose). The IC₅₀ value is half maximal inhibitory concentration of the inhibitor and it is commonly used as a measure of inhibitor effectiveness (Kim et al., 2005; Kazeem et al., 2013).

Alpha amylase inhibitor effectiveness of *B. abyssinica* extracts

The acetone and aqueous extracts inhibitory activities on the α -amylase were evaluated as described by Kazeem et al. (2013). Briefly, 0.5 μ l of α -amylase (0.5 mg/ml) was pre-mixed with 12.5 μ l of acetone and aqueous extracts at various concentrations (2.4 to 78.1 μ g/ml) (the range, from the lowest to the highest, which demonstrated the best α -amylase inhibitory activities during screening) and starch (12.5 μ l) as a substrate was added at 1% starch solution in phosphate buffer to start the reaction. The reaction was carried for 20 minutes at 37°C and terminated by adding 25 μ l of DNS reagent (96 mM 3,5-dinitrosalicylic acid, 5.31M sodium potassium tartrate in 2 M NaOH). The reaction mixture was then heated for 15 minutes at 100°C, allowed to cool and diluted with 200 μ l of water. The α -amylase activity was determined by measuring absorbance at 540 nm.

The IC₅₀ values were determined from dose-response curve of percentage inhibition versus aqueous and acetone extracts concentration and compared with the IC₅₀ of the synthetic inhibitor of α -amylase (acarbose) determined under similar conditions.

Apha-glucosidase inhibitor effectiveness of *B. abyssinica* fractions

The acetone and aqueous extracts inhibitory activities on the α -glucosidase were evaluated as described by Kazeem et al. (2013). Briefly, 50 μ l of α -glucosidase (1.0 Unit/ml) was pre-mixed with acetone and aqueous extracts at various concentrations (0.61 to 39.1 μ g/ml) (the range, from the lowest to the highest, which demonstrated the best α -glucosidase inhibitory activities during screening) and 5 mM pNPG as a substrate in phosphate buffer was added to the mixture to start the reaction. The reaction was incubated at 37°C for 30 minutes and stopped by addition of 50 μ l of 0.1 M Na₂CO₃. Alpha glucosidase activity was determined by measuring release of p-nitrophenyl α -D-glucoparanoside at 400 nm. IC₅₀ values were estimated from dose-response curves of percentage inhibition versus extract concentration and compared with the IC₅₀ of acarbose determined under similar conditions.

Modes of inhibition of *B. abyssinica* extracts on enzyme activity

The mode of inhibition of the plant extract was evaluated using the extract which showed the lowest IC₅₀ according to the method described by Ali et al. (2006).

Mode of inhibition of aqueous extract on α -amylase activity

Mode of inhibition of this plant against the α -amylase was evaluated using the aqueous extract which showed the lowest IC₅₀ (3.28 μ g/ml) by the method described by Ali et al. (2006). Briefly, 100 μ l of the aqueous extract (78.1 μ g/ml) extract was pre-incubated with 200 μ l of α -amylase for 15 minutes at 37°C in one set of tubes. In the other set of tubes, α -amylase was pre-incubated with 100 μ l of phosphate buffer, pH 6.9. Four hundred micro liters of 1% starch at increasing concentrations (0.88 to 14.62 mM) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 20 minutes at 37°C, and then boiled at 100°C for 15 minutes after addition of 200 μ l of DNS to stop the

reaction. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal plot ($1/v$ versus $1/[S]$) where v is reaction velocity and $[S]$ is substrate concentration was plotted. The type (mode) of inhibition of the aqueous extract on α -amylase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

Mode of inhibition of acetone extract on α -glucosidase activity

Mode of inhibition of this plant against the α -glucosidase was evaluated using the acetone extract which showed the lowest IC_{50} (4.27 $\mu\text{g/ml}$) as described by Ali et al. (2006) with some modifications. Briefly, 50 μl of the acetone (39.1 $\mu\text{g/ml}$) extract was pre-incubated with 100 μl of α -glucosidase for 30 minutes at 37°C in one set of tubes. In the other set of tubes, were pre-incubated with 50 μl of phosphate buffer, pH 6.9. Then, 50 μl pNPG at increasing concentrations (1 to 5 mM) was added to both sets of reaction mixtures to start the reaction. The mixtures were then incubated for 30 minutes at 37°C, and 500 μl of 0.1 M Na_2CO_3 was added to stop the reaction. This was further incubated at 37°C for 20 minutes. The amount of p-nitrophenol released was measured spectrophotometrically at 400 nm using a p-nitrophenol standard curve and converted to reaction velocities. A double reciprocal plot ($1/v$ versus $1/[S]$), where v is reaction velocity and $[S]$ is substrate concentration was plotted. The type (mode) of inhibition of the crude extract on α -glucosidase was determined by analysis of the double reciprocal plot using Michaelis-Menten kinetics. All the experiments were done in triplicate.

Data analysis

The experimental data was expressed as mean \pm standard deviation (SD) of the three replicates. Statistical analysis was done by using MINITAB program (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used to compare the data among the plant extracts and the control. $p < 0.05$ was considered statistically significant.

Results

The *in vitro* α -amylase inhibitory activities showed that aqueous extract of *B. abyssinica* had an appreciable inhibitory effect on α -amylase while acetone extract had a weak inhibitory effect. The inhibitory activity with the two plants fractions and the synthetic inhibitor (acarbose) was dose dependent from 2.4 to 78.1 $\mu\text{g/ml}$. At a dosage of 19.5 $\mu\text{g/ml}$, the aqueous extract showed 88% inhibition while acetone extract exhibited 55.8% which were significantly lower than the control. The aqueous extract exhibited significantly similar inhibitory activities with acarbose at 78.1 $\mu\text{g/ml}$ with 96.3% and 94.5% inhibition, respectively (Figure 1). The α -amylase inhibition effectiveness of the plant extracts were compared on the basis of the IC_{50} values. The highest inhibitory activity was achieved with the aqueous extract (IC_{50} value of 3.28 $\mu\text{g/ml}$) which was significantly lower than the positive control (acarbose) (Table 1).

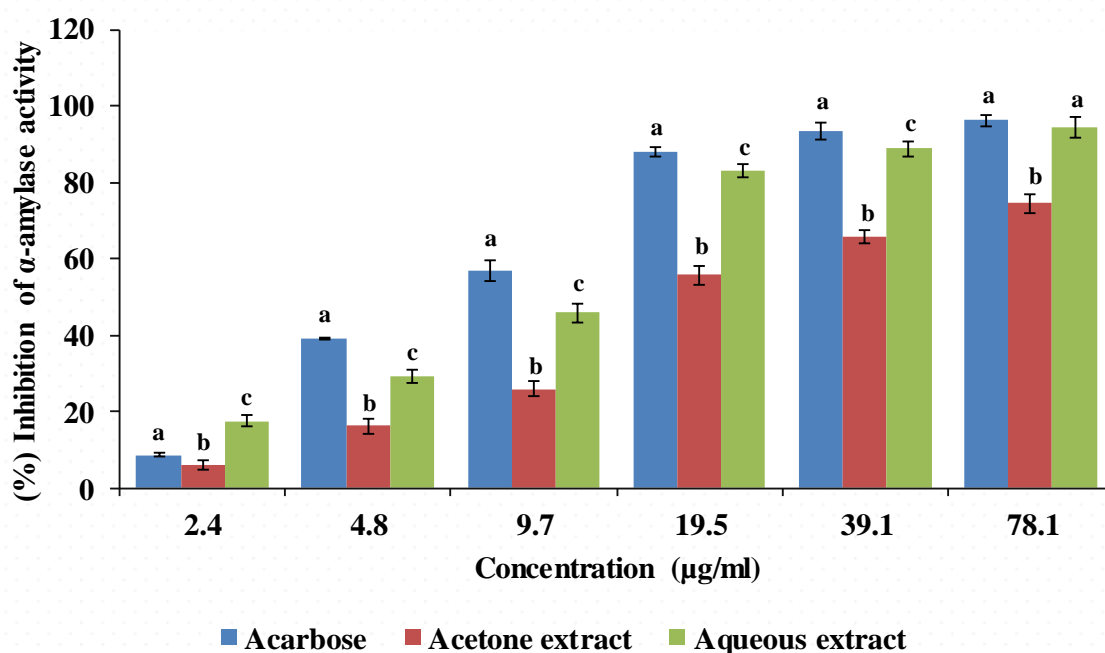


Figure 1: Effects of acetone and aqueous whole plant extracts of *B. abyssinica* on α -amylase activity. Data are presented as means \pm SD of three replicates. The inhibitory activities for each concentration in the different extracts having different letters are significantly different from each other ($p < 0.05$).

Table 1: Inhibitory effects of acetone and aqueous whole plant extracts of *B. abyssinica* on the α -amylase activity.

Extract/Control	IC ₅₀ ($\mu\text{g/ml}$)
	α -amylase
Acetone	4.97 \pm 0.12 ^a
Aqueous	3.28 \pm 0.08 ^b
Acarbose	1.79 \pm 0.17 ^c

IC₅₀ is defined as the concentration of inhibitor sufficient to inhibit 50% of the enzyme activity. Data are presented as means \pm SD of three replicates. Values along a column with different superscripts are significantly different ($p < 0.05$).

The inhibition mode of action for aqueous extract against α -amylase was therefore investigated. The Lineweaver–Burk plots analysis revealed that α -amylase had a Michaelis-Menton constant (K_m) of 1.29 mM for starch as a substrate and V_{max} of 0.76 mMmin⁻¹. The apparent V_{max} values were reduced from 0.76 mM/min to 0.38 mM/min in the presence of aqueous extract of this plant (78.1 μ g/ml) while the K_m remained unchanged at 1.29 mM for starch (Figure 2). Thus, the aqueous extract of this plant displayed patterns of non-competitive inhibition.

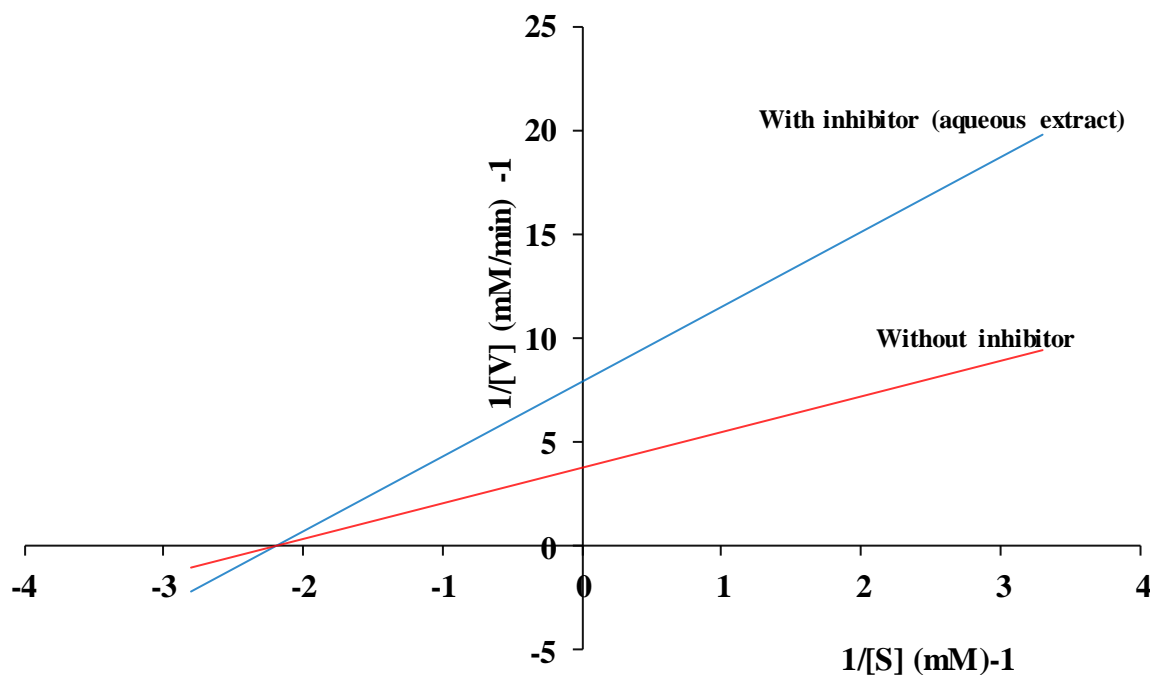


Figure 2: Lineweaver-Burk double reciprocal plots for kinetic analysis of the reaction of α -amylase with starch (mM) used as substrate in the absence and presence of aqueous whole plant extract of *B. abyssinica* (78.1 μ g/ml).

The plant extracts inhibitory potentials against α -glucosidase were evaluated at concentrations ranging from 0.6 to 39.1 μ g/ml. The aqueous extract showed significantly

weaker inhibitory activities as compared to acetone extract and acarbose at all different concentrations, in a dose dependent manner, except at 0.6 $\mu\text{g/ml}$. At 4.9 $\mu\text{g/ml}$, aqueous extracted exhibited 22.2% inhibition on the enzyme activity against 38.2% and 64.9% with acetone extract and acarbose, respectively. This trend was observed until at 19.53 $\mu\text{g/ml}$. At 39.1 $\mu\text{g/ml}$, acetone extract and acarbose revealed significantly similar inhibitory activities on the enzyme, which were significantly higher than that observed with the aqueous extract (Figure 3). The estimated IC_{50} values showed that the acetone extract exhibited the highest α -glucosidase inhibitory activities with an IC_{50} value of 4.28 $\mu\text{g/ml}$, though significantly lower than the standard drug (Table 2). The inhibition mode of acetone extract against α -glucosidase was then evaluated.

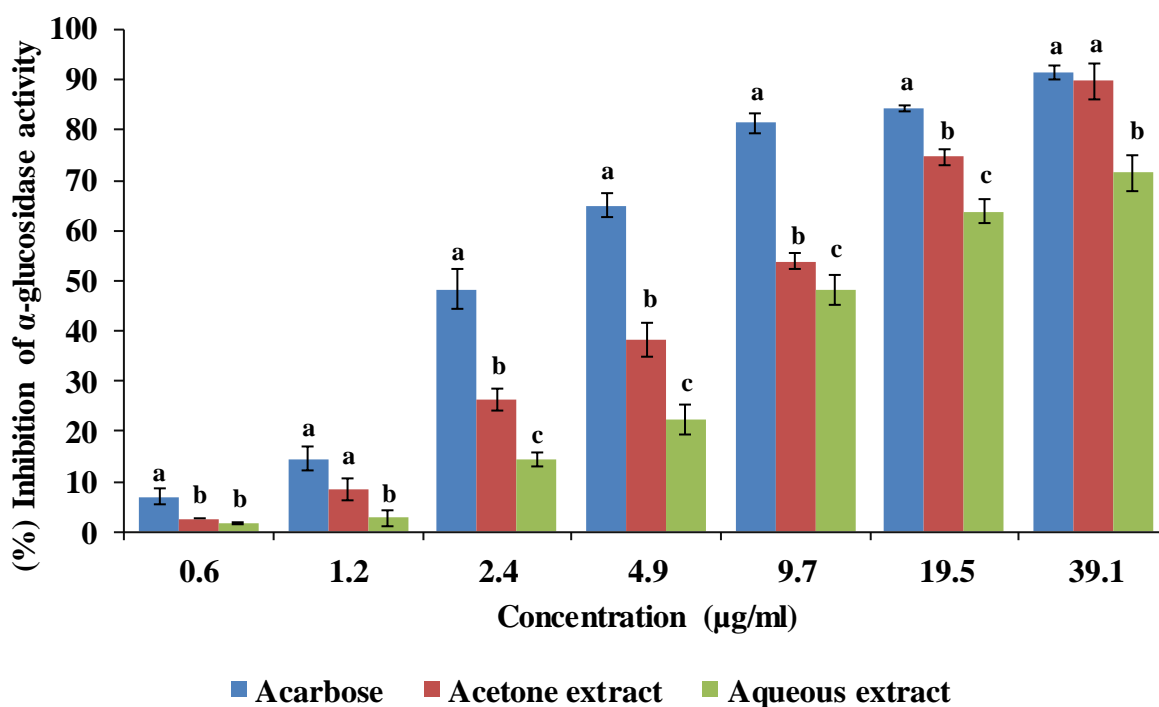


Figure 3: Effects of acetone and aqueous whole plant extracts of *B. abyssinica* on α -glucosidase activity. Data are presented as means \pm SD of three replicates. The inhibitory activities for each concentration in the different extracts having different letters are significantly different from each other ($p < 0.05$).

Table 2: Inhibitory effects of acetone and aqueous whole plant extracts of *B. abyssinica* on the α -glucosidase activity.

Extract/Control	IC ₅₀ (μ g/ml)
	α -glucosidase
Acetone	4.28 \pm 0.02 ^a
Aqueous	6.35 \pm 0.19 ^b
Acarbose	3.56 \pm 0.14 ^c

IC₅₀ is defined as the concentration of inhibitor sufficient to inhibit 50% of the enzyme activity. Data are presented as means \pm SD of three replicates. Values along a column with different superscripts are significantly different ($p < 0.05$).

The Lineweaver–Burk plots analysis revealed that α -glucosidase had a K_m of 5.6 mM for pNPG as a substrate and V_{max} of 0.2 mM/min. In the presence of the acetone extract (39.1 μ g/ml) (inhibitor), the apparent V_{max} values decreased from 0.2 mM/min to 0.1mM/min while the K_m changed from 5.6 mM to 4.6 mM for pNPG (Figure 4). This implies that this species displayed patterns of near competitive inhibition.

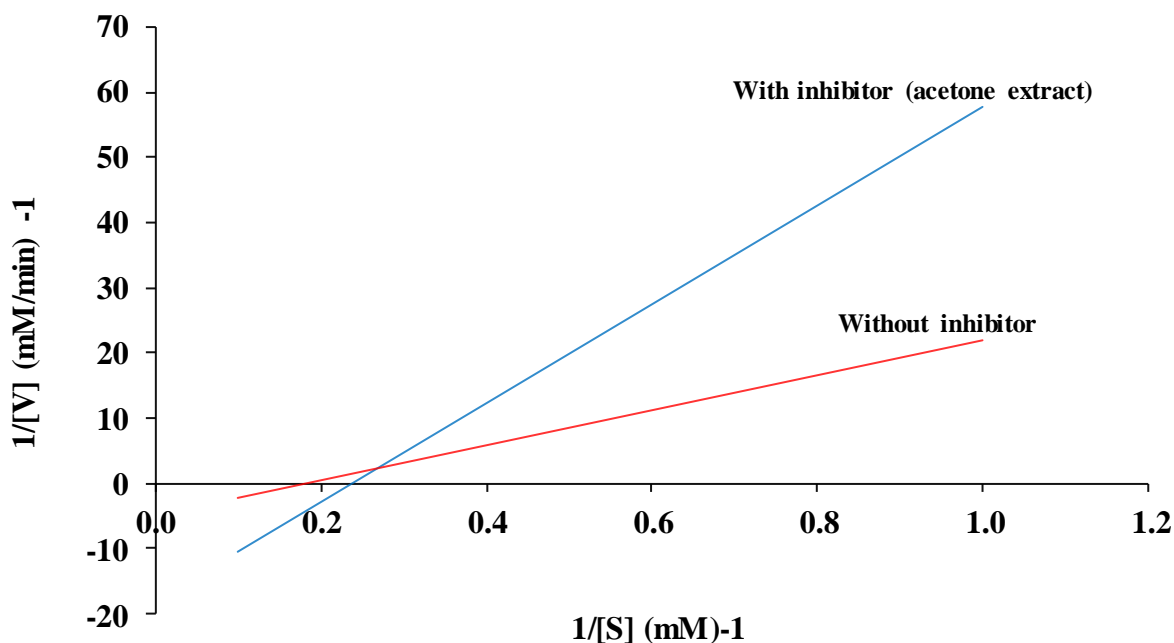


Figure 4: Lineweaver-Burk double reciprocal plots for kinetic analysis of the reaction of α -glucosidase with pNPG (mM) used as substrate in the absence and presence of acetone whole plant extract of *B. abyssinica* (39.1 μ g/ml).

Discussion

The most challenging goal in the management of diabetes mellitus is achieving blood glucose levels as close to normal levels physiologically as possible (Nathan and Russell, 2013). The rise in postprandial hyperglycaemia is one of the earliest metabolic abnormalities detectable in diabetic patients (Blaak et al., 2012). Failure to arrest prolonged hyperglycaemia leads to development of microvascular and macrovascular complications of diabetes mellitus (Chang et al., 2013).

The research and use of complementary medicines for the management of diabetes mellitus has been recommended by WHO (Konate et al., 2014). The inhibition of key enzymes linked to diabetes mellitus, such as α -amylase and α -glucosidase, has been considered to be

effective strategy to control blood glucose using herbal therapy (Konate et al., 2014). These enzymes have been recognized as therapeutic targets for modulation by these antidiabetic agents (Blaak et al., 2012).

Natural products remedies with this type of modulation are particularly attractive as side effects are minimal and the therapies are well tolerated compared to the present oral hypoglycemic agents (Picot et al., 2014). Thus, in the present study, *B. abyssinica* was evaluated for these activities.

The results show that the acetone and aqueous whole plant extracts of this plant have inhibitory potentials on the two model enzymes. The aqueous extract showed remarkable α -amylase inhibition than the acetone extract, while the acetone extract showed more inhibition on α -glucosidase than the aqueous extract. The difference in activity could be due to the different compounds present as a result of different solvents used in this study. Different solvents have varying extraction potentials (Dai and Mumper, 2010).

Acarbose, the positive control used in this study, inhibited the activity of α -glucosidase with an IC_{50} value estimated at 3.56 μ g/ml lower than α -amylase activity inhibition (Table 1). This finding is consistent with other literature reports, where acarbose was found to exhibit little or no inhibition on α -glucosidase activity (Anam et al., 2009; Shai et al., 2010). This indicates that α -glucosidase is highly insensitive to acarbose hence there is need to search for novel inhibitors.

In the present study, the aqueous extract of this plant displayed patterns of non-competitive inhibition on the α -amylase activity. This suggests that the active component(s) of the extract binds to a site other than the active site of the enzyme, and combines with either the free

enzyme or the enzyme substrate complex possibly interfering with the action of both. This reduces the inhibitory potential on the enzyme activity (Shai et al., 2010; Tu et al., 2013).

Furthermore, the acetone extract of this species showed a near competitive inhibition pattern on the α -glucosidase activity. This implies that the bioactive compound(s) competes with the substrate for binding to the active site of the α -glucosidase, thereby preventing or slowing down the breakdown of the starch (oligosaccharides) to disaccharides hence reduce the gastrointestinal glucose production and absorption, thereby arresting postprandial hyperglycemia (Kazeem et al., 2013). This further suggests that the acetone extract might possess active ingredients which can serve as potential candidate(s) for α -glucosidase blockers (inhibitors).

In conclusion, it is obvious from the data obtained from this study that the acetone and aqueous whole plant extracts of *B. abyssinica* have inhibitory properties to α -amylase and α -glucosidase activity. This could be only part of mechanism of action of this plant hence there is need for further studies to access other probable pathways and specific mechanisms the species could possess. Future studies could also focus on the isolation of the inhibitory components of this plant and their potential as novel drugs to control diabetes mellitus.

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CHAPTER 4

***IN VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF THE ESSENTIAL OIL, ACETONE AND AQUEOUS WHOLE PLANT EXTRACTS OF *BULBINE ABYSSINICA*.**

CHAPTER FOUR

***In vitro* antioxidant and anti-inflammatory activities of the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*.**

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Introduction

Over the years, numerous studies have demonstrated that oxidative stress, mediated mainly by hyperglycemia-induced generation of free radicals, contributes to the development and progression of diabetes and related conditions such as inflammation. Therefore, it has become clear that ameliorating oxidative stress through treatment with antioxidants has become an effective strategy for controlling diabetes mellitus and reducing the complications associated with it (Johansen et al., 2005).

Several studies have shown that dietary antioxidants such as vitamins ameliorate the diabetic state and retards the development of complications in diabetes (Nishikawa and Araki, 2013). Thus, antioxidants are reducing agents that can limit cellular oxidative stress hence attenuating inflammation and serving as antidiabetic agents (Umapathy et al., 2010).

Human cells have an array of protective mechanisms that prevent production of free radicals and attenuate oxidative damage (Poljsak et al., 2013). These mechanisms include; release of enzymatic and non-enzymatic antioxidants such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, ascorbic acid and tocopherol (Poljsak et al., 2013). The protective roles of these enzymes may be disrupted as a result of various pathological processes and thereby causing damage to the cells. Therefore, the cells also offer protection against inflammation via inhibiting protein denaturation agents and protection against membrane lysis (Shruthi et al., 2012). Synthetic antioxidants (such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)) and Non-steroidal inflammatory drugs (NSAIDs) (such as Diclofenac sodium and Aspirin) are commercially available and currently used (Shruthi et al., 2012). However, these drugs have side effects

such as dizziness, heartburn and diarrhea. Therefore, this has led to seeking alternative medicine from plant remedies (Shruthi et al., 2012).

Natural products of plant origin have been proposed as a potential source of natural antioxidants with strong activity. This activity is mainly due to the presence of phenolic or nitrogen containing compounds and carotenoids (Erdemoglu et al., 2006). A vast majority of plants used in traditional medicine in South Africa have currently not been evaluated for their antioxidant potential. One of such plants is *B. abyssinica*.

B. abyssinica is among the most used species in South Africa as a folk remedy for the treatment of diabetes mellitus (Oyedemi et al., 2009). However, there is no scientific evidence available in literature to show the mechanism of action as an antidiabetic agent and to support its use in the traditional medicine.

Before the commencement of this work, to the best of my knowledge, there was no information in scientific literature on the *in vitro* antioxidant and anti-inflammatory activities of the essential oil, aqueous and acetone whole plant extracts of *B. abyssinica*. Therefore, this study was aimed at providing information on these pharmacological activities of this plant.

Materials and Methods

Plant materials

The whole plant parts of *B. abyssinica* including the leaves, flowers, stems and roots were collected and authenticated as described in Chapter 3.

Preparation of plant extracts

Some of the plant samples were air-dried, ground to homogeneous powder and extracted using acetone and water. The acetone and aqueous extraction was done as described in

Chapter 3. The resulting extracts were reconstituted with their respective solvents to give the desired concentrations used in the study.

The essential oil was extracted from fresh plant samples using hydro-distillation method, according to the procedure of the European Pharmacopoeia (2002). Briefly, 250 g of the whole plant samples were hydro-distilled for 3 h in an all-glass Clevenger. Heat was supplied to the heating mantle (50°C), and the essential oil was extracted with 4 liters of water for 3 h (until no more essential oil was coming out). The average percentage yield was 0.4%. The essential oils were dissolved with appropriate vehicles for further *in vitro* bioassay activities.

Chemicals and reagents used

The chemicals used include; 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Vanillin, Butylated hydroxyl toluene (BHT), Rutin, Potassium persulphate, Sodium nitroprusside ($\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]2\text{H}_2\text{O}$), Sulfanilic acid, Glacial acetic acid (CH_3COOH), Gallic acid, Tannic acid, Ferric chloride (FeCl_2), Ascorbic acid, Folin-Ciocalteu reagent, Sodium carbonate (Na_2CO_3), Aluminium chloride (AlCl_3), Potassium acetate ($\text{CH}_3\text{CO}_2\text{K}$), Potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$], Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), Diclofenac sodium, Hydrochloric acid (HCL), Bovine serum albumin (BSA), Sodium chloride (NaCl), Sodium dihydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4) and Dimethyl sulfoxide (DMSO). These chemicals were purchased from Merck and Sigma-Aldrich, Gauteng, South Africa. All the chemicals used in this study were of analytical grade.

***In vitro* antioxidant analysis**

The antioxidant activities of the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* were evaluated using DPPH, nitric oxide, reducing power, hydrogen peroxide, ABTS and lipid peroxidation inhibitory assays.

Ferric reducing power assay

The reducing power of the plant fractions was determined by the method of Wintola and Afolayan (2011) with slight modifications. Briefly, different concentrations (0.025 to 0.5 mg/ml) of extracts (0.5 ml) were mixed with 0.5 ml 0.2 M phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexa-cyanoferrate, followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml 10% Trichloroacetic acid was added to terminate the reaction. The supernatant of the solution (1 ml) was mixed with 1 ml of distilled water and 0.1 ml 0.01% FeCl₃ solution added. The reaction mixture was left for 10 min at room temperature and the absorbance measured at 700 nm against the appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power.

DPPH radical scavenging activity assay

The method of Wintola and Afolayan (2011) was used for the determination of DPPH free radical scavenging activity. Briefly, a solution of 0.135 mM DPPH radical in methanol was prepared. A 1 ml of this solution was mixed with 1.0 ml of each plant fraction (0.025 to 0.5 mg/ml) and standard drugs (BHT, rutin and vitamin C) (0.025 to 0.5 mg/ml). The reaction mixture was then vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The actual decrease in absorbance was measured against that of the control. The scavenging ability of the plant extract was then calculated using this equation:

DPPH Scavenging activity (%) = [(Abs control – Abs sample)/(Abs control)]×100;

Where; Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample (sample or standard).

Nitric oxide scavenging activity assay

The modified method described by Oyedemi et al. (2010) was used to determine the nitric oxide radical scavenging activity. A volume of 2 ml of 10 mM of sodium nitroprusside prepared in 0.5 mM phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant fractions, rutin and vitamin C individually to make different concentrations from 0.025 to 0.5 mg/ml. The mixture was incubated at 25° C for 150 min. Then, 0.5 ml of incubated solution was then mixed with 0.5 ml of Griess reagent (1.0 ml sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid) at room temperature for 5 min with 1 ml naphthylethylenediamine dichloride (0.1% w/v). The mixture was incubated at room temperature for 30 min and absorbance taken at 540 nm. The amount of nitric oxide radicals inhibited by the plant fraction/standard drugs was calculated using the following equation;

NO radical scavenging activity (%) = [(Abs control – Abs sample)/(Abs control)] ×100, where; Abs control is the absorbance of NO radicals + methanol; Abs sample is the absorbance of NO radical + extract or standard.

Hydrogen peroxide (H₂O₂) radical scavenging activity assay

The H₂O₂ inhibition activity of the plant fractions was assessed by the method of Ilhami et al. (2005). Briefly, a solution of 4 mM H₂O₂ was prepared in phosphate buffer (0.1 M; pH 7.4) and incubated for 10 min. One milliliter of each plant extract (0.025 to 0.5 mg/ml) was added to a 0.6 ml of hydrogen peroxide solution. The absorbance of the hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer

solution without hydrogen peroxide. The positive controls used were BHT (0.025 to 0.5 mg/ml) and vitamin C (0.025 to 0.5 mg/ml). The percentage scavenging of hydrogen peroxide in the samples was calculated using the following formula:

$$\text{H}_2\text{O}_2 \text{ inhibition capacity (\%)} = [1 - (\text{H}_2\text{O}_2 \text{ cons. of sample} / \text{H}_2\text{O}_2 \text{ cons. of blank})] \times 100.$$

2, 2'-Aazino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The method described by Otang et al. (2012) was adopted for the determination of ABTS scavenging activity. Briefly, the stock solutions including; 7 mM ABTS solution and 2.4 mM potassium persulfate solution were prepared. The working solution was then prepared by mixing the two stock solutions in equal proportions and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS⁺ solution with 60 ml of methanol to obtain an absorbance of 0.708 ± 0.001 units at 734 nm using the spectrophotometer. The plant fractions (1 ml) and their controls were allowed to react with 1 ml of the ABTS⁺ solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS⁺ scavenging capacity of the extract was then compared with that of the standards. The percentage inhibition was then calculated as follows;

$$\text{Inhibition \%} = [(A \text{ blank} - A \text{ sample}) / A \text{ blank}] \times 100, \text{ where;}$$

A blank is the absorbance of ABTS radical + methanol used as control; A sample is the absorbance of ABTS radical + sample extract/standard.

Lipid peroxidation scavenging activity assay

The inhibition of lipid peroxidation in the rat liver homogenate was determined using a modified thiobarbituric acid reactive species (TBARS) assay as described by Murugan and Parimelazhagan (2014). The liver homogenate (0.5 ml, 10% in distilled water, v/v) and 0.1

ml of each plant fractions/controls were mixed separately in a test tube and the volume was made up to 1 ml by adding distilled water. Then, 0.05 ml FeSO₄ (0.07 M) was added to the above mixture and incubated for 30 min to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA (w/v) in 1.1% sodium dodecyl sulfate and 0.05 ml 20% TCA was added, vortexed and then heated in a boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. For the blank, 0.1 ml of distilled water was used in place of the plant sample. Inhibition (%) of lipid peroxidation was calculated using the equation:

% Inhibition = [(Control OD - Sample OD)/Control OD] X 100. All antioxidant assays were done in triplicates.

***In vitro* anti-inflammatory activity**

Protein denaturation method

The protein denaturation assay was determined using a modified method as described by Murugan and Parimelazhagan (2014). Briefly, the reaction mixture (0.5 ml; pH 6.3) consisted of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of distilled water. The pH was adjusted to 6.3 using a small amount of 1 N HCL. A 1 ml of essential oil or acetone or aqueous extract (with final concentrations of 0.1 to 0.5 mg/ml) was added to the reaction mixture. These were incubated at 37°C for 30 min and then heated at 57°C for 5 min. After cooling the samples, 2.5 ml of phosphate buffer solution (pH 6.4) was added. Turbidity was measured spectrophotometrically at 660 nm. For the negative control, 0.05 ml distilled water and 0.45 ml of bovine serum albumin were used. Diclofenac sodium with the

final concentration of 100, 200, 300, 400, 500 µg/ml was used as reference drug. The percentage inhibition of protein denaturation was calculated by using the following formula;

$$\text{Percentage Inhibition (\%)} = [(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100.$$

Membrane stabilizing activity

Hypotonic solution-induced rat erythrocyte haemolysis

The rat erythrocyte cells were prepared using the method described by Majumder et al. (2008). Briefly, the whole blood was obtained with heparinized syringes from a rat through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged after each wash for 10 min at 13,000 rpm (Majumder et al., 2008).

Membrane stabilizing activity of the extracts was assessed using hypotonic solution-induced rat erythrocyte haemolysis method as described by Majumder et al. (2008). Briefly, the test sample consisted of stock erythrocyte (RBC) suspension (0.5 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the plant fraction or standard drug at concentrations ranging from 0.1 to 1 mg/ml. The control sample consisted of 0.5 ml of RBC mixed with hypotonic-buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 13,000 rpm. The absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated as follows;

$$\% \text{ Inhibition of haemolysis} = 100 \times [\text{OD1} - \text{OD2} / \text{OD1}];$$

Where: OD1 is the Optical density of hypotonic-buffered saline solution alone and OD2 is the Optical density of test sample in hypotonic solution.

The activity was expressed as 50% inhibitory concentration (IC_{50}). IC_{50} values were estimated from the best-fit line obtained by linear regression analysis of the percentage scavenging activity/ inflammation inhibitory activity versus the concentration. The lower the IC_{50} value, the higher the antioxidant or anti-inflammatory activity (Park et al., 2014).

Data analysis

The experimental data was expressed as mean \pm standard deviation (SD) of the three replicates. Statistical analysis was done by using MINITAB program (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used to compare the data among the plant fractions with the controls. $p < 0.05$ was considered statistically significant.

Results

The essential oil, acetone and aqueous extracts of this species showed remarkable percentage inhibition activity in all the free radical scavenging *in vitro* models used in the present study. The antioxidant activities of the plant fractions were estimated from their ability to reduce Fe^{3+} to Fe^{2+} which was concentration dependent on the extracts and the reference standard. The trend of the reducing potential of the all the extracts was significantly lower than that of BHT followed by Vitamin C except at 0.05 mg/ml in which essential oil inhibitory activity was significantly similar to BHT (Figure 1). The IC_{50} values show that the essential oil had the highest Fe reducing activity, followed by aqueous extract, and then acetone extract. Only essential oil had more reducing potential than BHT and Vitamin C (Table 1).

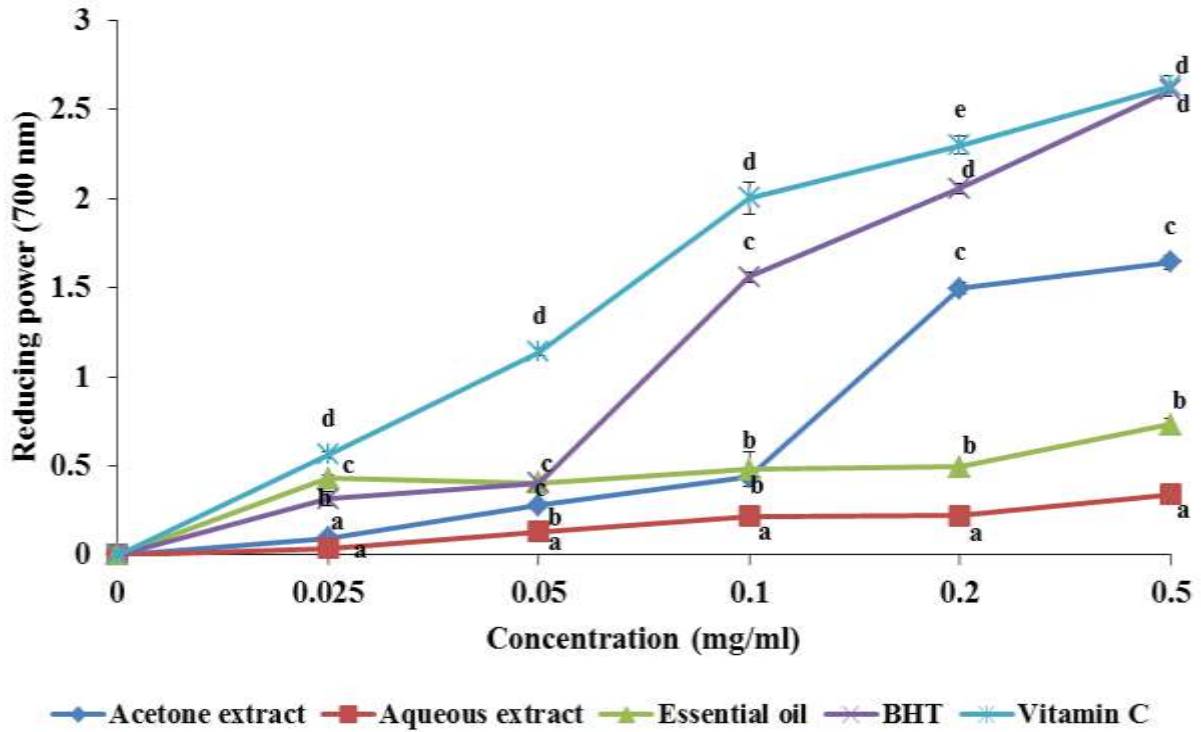


Figure 1: Reducing power of BHT, Vitamin C, essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Line points with different letter superscript within the same concentration are significantly different ($P < 0.05$).

Table 1: Scavenging and inflammation inhibitory activities of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*.

Activity	A		B		C		D		E		F		G		H	
Samples	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b	IC ₅₀ ^a	R ² ^b
Acetone extract	1.25	78.3	0.19	79.6	0.23	89.9	0.04	88.4	0.18	83.6	0.14	86.2	0.22	74.3	0.51	90.1
Aqueous extract	0.19	79.7	0.26	99.7	0.14	85.9	0.24	99.8	1.61	92.2	0.21	87.8	0.33	71.5	0.33	72.2
Essential oil	0.09	80.5	0.2	84.2	0.16	86.3	0.17	86.3	2.17	94.4	0.21	87.1	0.23	73.37	0.47	86.3
BHT	0.18	88.7	0.2	90.4	–	–	0.25	99.4	2.04	84.8	0.16	57.3	–	–	–	–
Vitamin C	0.12	85.3	0.07	89.7	0.09	84.9	0.22	93.1	2.21	96.8	–	–	–	–	–	–
Rutin	–	–	0.26	81.4	0.23	91.1	–	–	–	–	0.18	81.3	–	–	–	–
Gallic acid	–	–	–	–	–	–	–	–	1.67	66.7	–	–	–	–	–	–
Diclofenac sodium	–	–	–	–	–	–	–	–	–	–	–	–	0.31	91.9	0.48	81.2

The letters represents; **A**=Reducing power; **B**=DPPH scavenging activity; **C**=Nitric oxide scavenging activity; **D**=Hydrogen peroxide scavenging activity; **E**= Lipid peroxide scavenging activity; **F**= ABTS scavenging activity; **G**= Protein denaturation inhibitory activity and; **H**= Membrane lysis inhibitory activity. **a**: IC₅₀ is defined as the concentration (mg/ml) sufficient to obtain 50% of a maximum scavenging capacity; **b**: coefficient of determination; values obtained from regression lines with 95% confidence level and -: Values not determined.

The results from a series of concentration ranging from 0.025 to 0.5 mg/ml were used to determine the concentration required to attain 50% DPPH radical scavenging effect (IC₅₀). A lower IC₅₀ value indicates higher scavenging activity (Park et al., 2014). The IC₅₀ values of the tested extracts/standards were in the order: Vitamin C < Acetone extract < BHT= Essential oil< Rutin =Aqueous extract (Table 1). Only the acetone extract exhibited a stronger scavenging activity than the essential oil in a dose-dependent manner. Though the essential oil had a significantly weaker scavenging activity (about 8-9 times fold) than the standards from 0.1 to 0.5 mg/ml, the activity was dose-dependent (Figure 2).

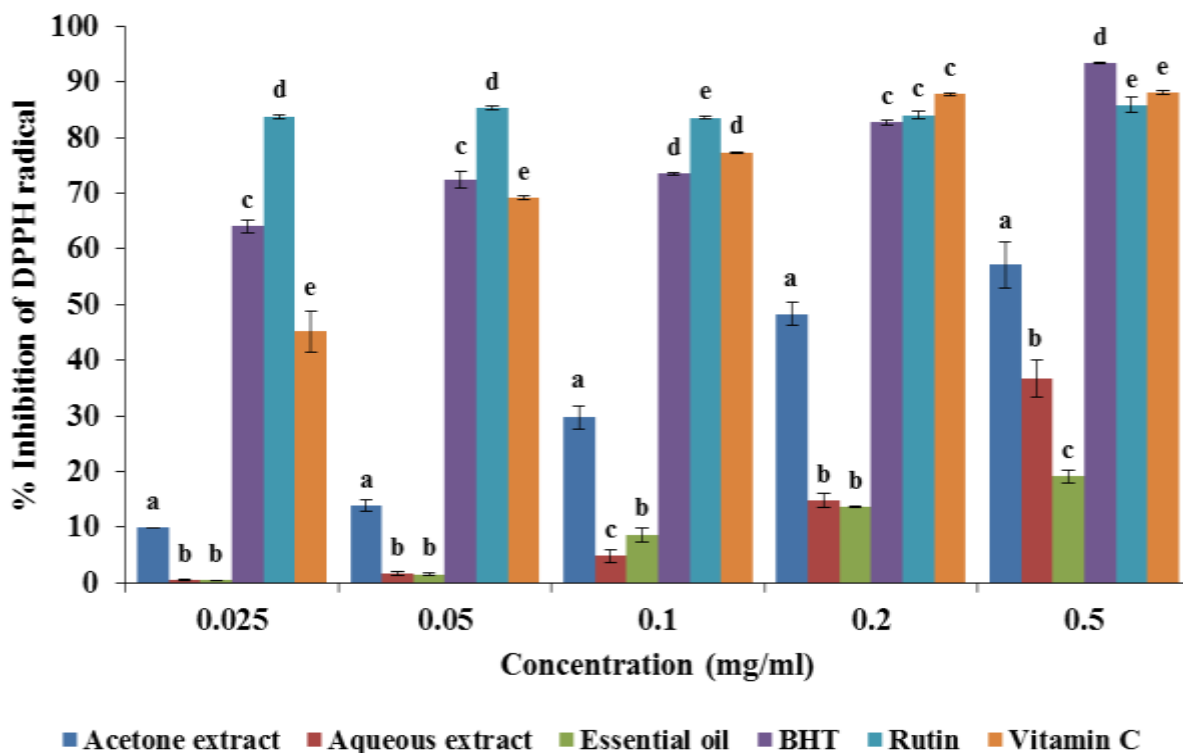


Figure 2: DPPH radical scavenging activity of BHT, rutin, Vitamin C, essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$).

The nitric oxide radical scavenging activity of the plant species was extrapolated as the percentage inhibition of nitric oxide. The scavenging activities were concentration dependent with aqueous extract, essential oil and Vitamin C from 0.025 to 0.5 mg/ml (Figure 3). However, the acetone extract exhibited significant similar high activity as the rutin standard. The IC₅₀ value determination indicates that the scavenging activity was significantly higher with Vitamin C, followed by aqueous extract, essential oil and then acetone extract and rutin (Table 1).

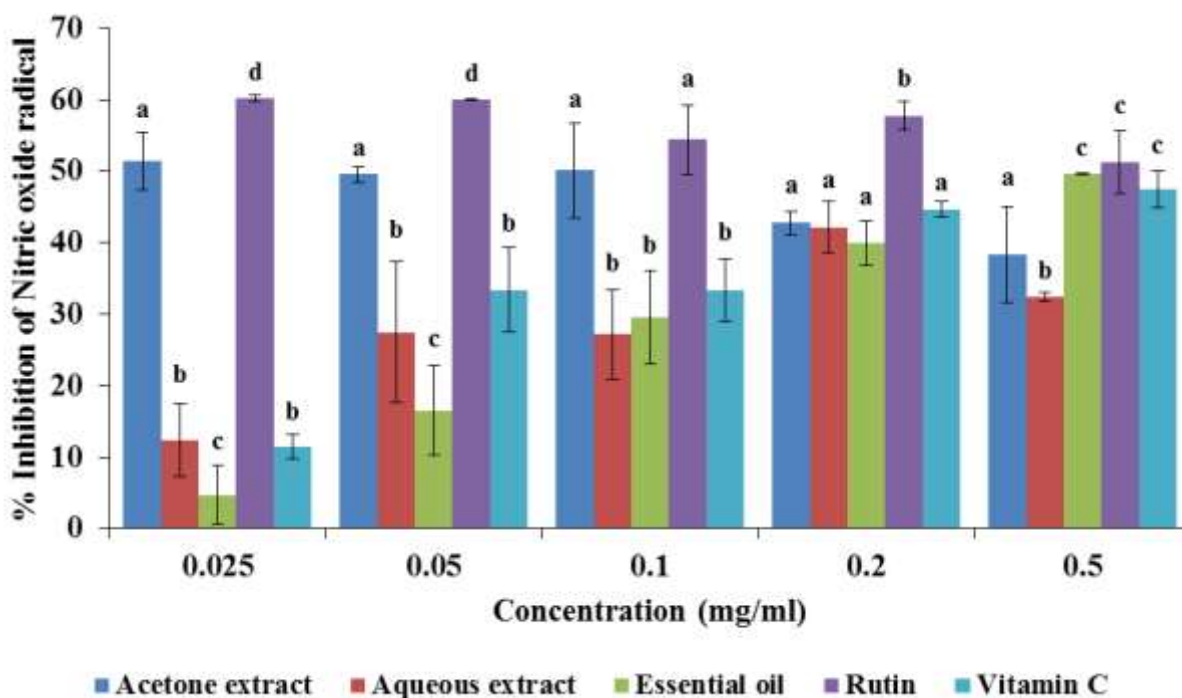


Figure 3: Nitric oxide radical scavenging activity of rutin, Vitamin C, essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$).

The IC₅₀ values for H₂O₂ scavenging activity for acetone extract, essential oil, aqueous extract, BHT and Vitamin C were 0.04, 0.17, 0.24, 0.25 and 0.22 mg/ml, respectively (Table 1). The H₂O₂ scavenging activity of both plant extracts and standards decreased with increasing concentration. The acetone extract showed significantly higher scavenging potential than the essential oil and aqueous extract in all concentrations, except at 0.05 mg/ml. Only the scavenging activity of aqueous and acetone extracts were significantly higher than those of the standards. The scavenging activity of the essential oil was similar to BHT and Vitamin C at 0.05 and 0.1 mg/ml (Figure 4).

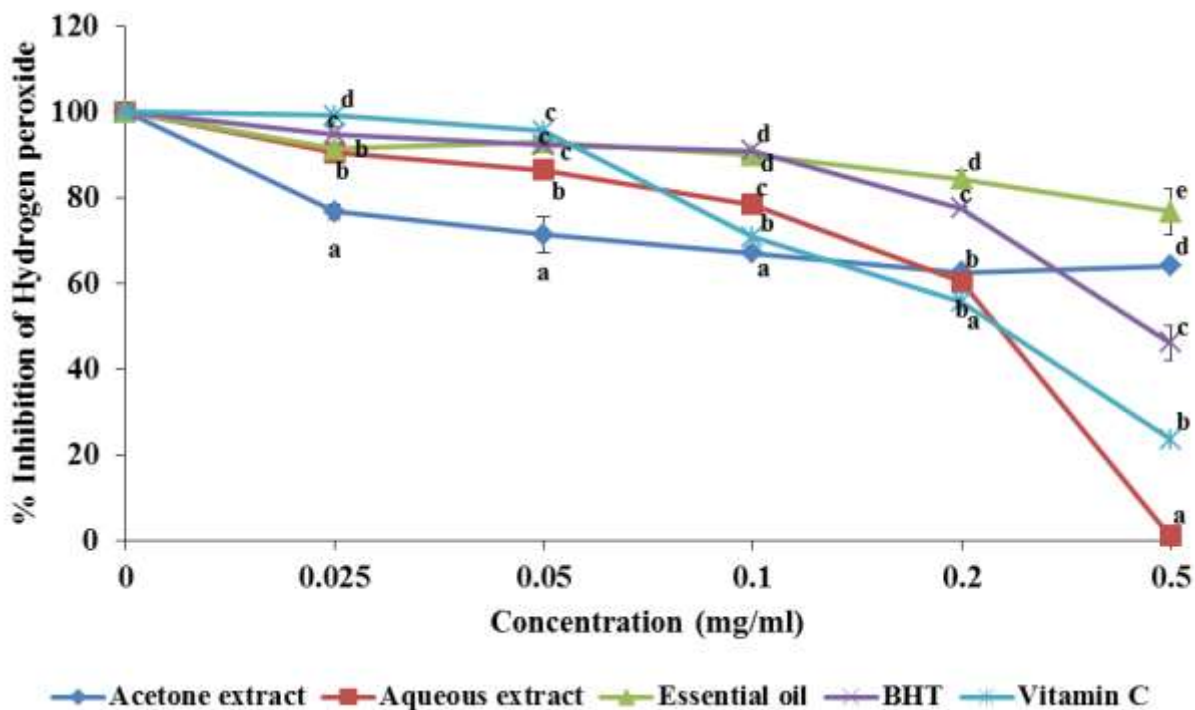


Figure 4: Hydrogen peroxide radical scavenging activity of BHT, Vitamin C, essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Line points with different letter superscript within the same concentration are significantly different ($P < 0.05$).

The percentage inhibition of ABTS radical by the species is shown in Figure 5. The percentage inhibition of ABTS by the plant fractions and the standards was concentration dependent and compared favorably with both BHT and rutin. The acetone extract's activity was significantly higher than the two standards. The essential oil and aqueous extract showed significantly similar inhibitory activities which were lower than the two controls. The IC₅₀ values of the plant fractions/standards were in the increasing order: Acetone extract < BHT < Rutin < Aqueous extract = Essential oil (Table 1).

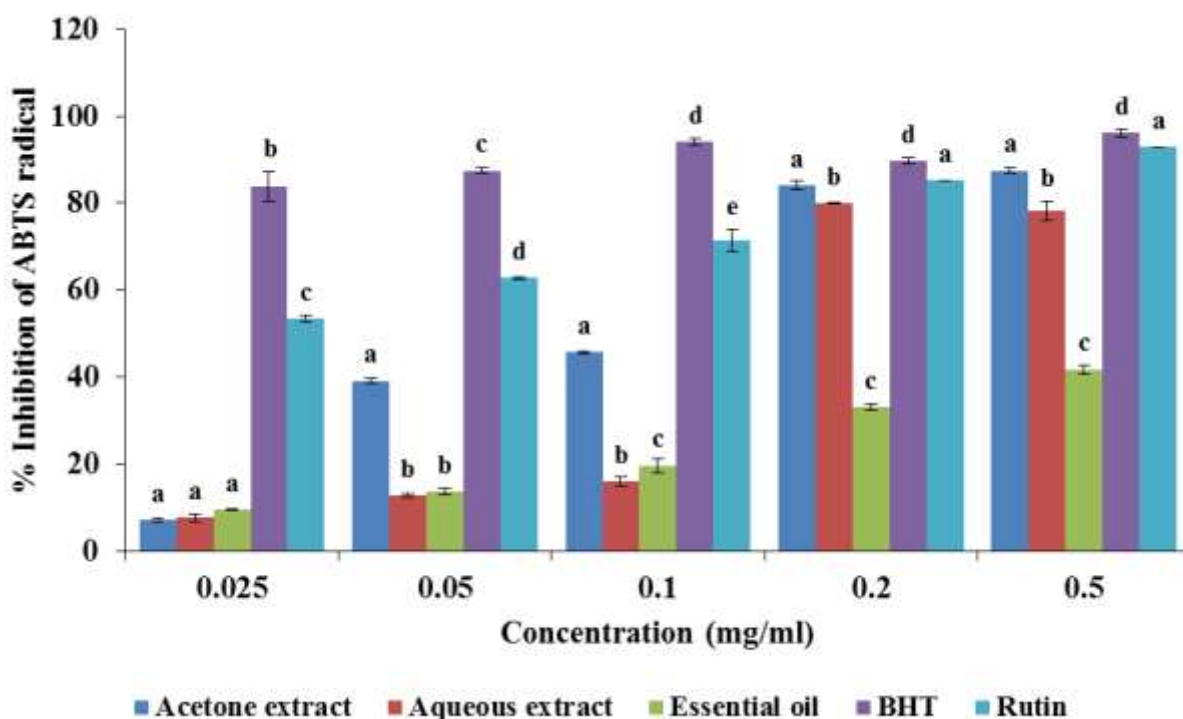


Figure 5: ABTS radical scavenging activity of BHT, rutin, essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$).

The scavenging of lipid peroxides by *B. abyssinica* extracts, BHT, gallic acid and Vitamin C is presented in Figure 6. The percentage inhibition of lipid peroxides by the plant fractions and the standards was recorded in significantly increasing order: Vitamin C > Essential oil > BHT > Gallic acid > Aqueous extract > Acetone extract. The estimated IC₅₀ values indicate that acetone extract had the highest inhibitory activity followed by aqueous extract and then the essential oil (Table 1).

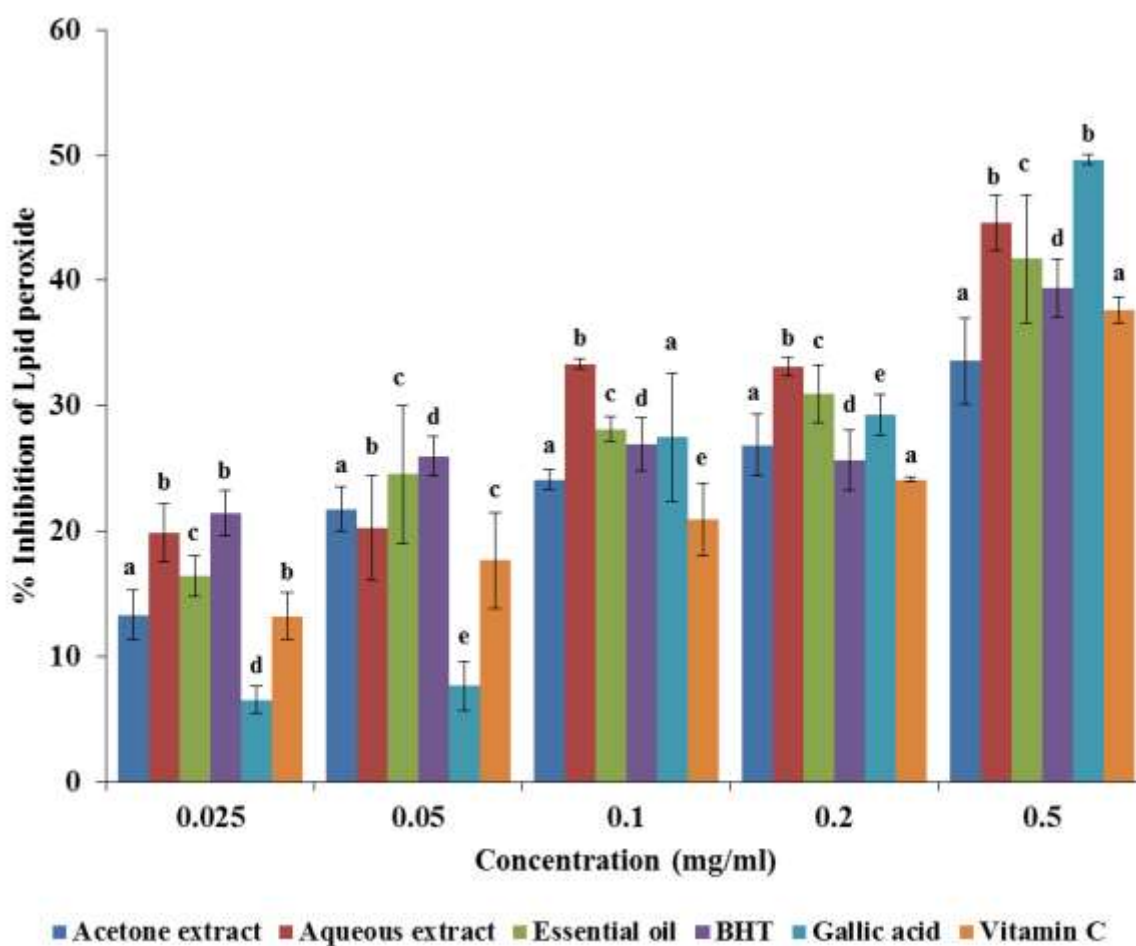


Figure 6: Lipid peroxidation scavenging activity of BHT, gallic acid, Vitamin C, essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$).

The results show that the free radical scavenging activities of these plant fractions varies according to free radical species. All these fractions showed high activities with acetone extract exhibiting the highest levels in all assay models except with ferric reducing power and nitric oxide scavenging ability. The aqueous extract exhibited the highest ferric reducing power and nitric oxide radicals while the essential oil exhibited the highest ferric reducing power, DPPH and nitric oxide scavenging ability.

The *in vitro* anti-inflammatory activity is represented in Table 1, 2 and 3. Acetone extract protected the albumin from denaturation in a dose dependent manner whose activity was significantly lower than the standard drug at all concentrations. The estimated IC₅₀ values for acetone extract and standard drug were 215 and 312 µg/ml, respectively (Table 1). The aqueous extract also showed significant protein denaturation inhibition (IC₅₀ value of 330 µg/ml) with increase in the concentration (100 to 300 µg/ml) when compared to the standard drug. However, it showed a significant decrease from 400 µg/ml (Table 2).

The protein denaturation activity of the essential oil significantly increased with increase in concentration with an IC₅₀ value of 231 µg/ml. This inhibitory activity was significantly lower than the standard drug's activity (IC₅₀ value of 312 µg/ml), except with 300 and 500 µg/ml (Table 2).

Table 2: Protein denaturation inhibitory activity of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*.

Concentration ($\mu\text{g/ml}$)	Acetone extract	Aqueous extract	Essential oil	Diclofenac sodium
500	85.09 ± 0.76^a	67.98 ± 11.50^b	97.81 ± 2.74^c	98.68 ± 2.28^c
400	79.39 ± 0.75^a	78.95 ± 2.63^a	88.16 ± 1.32^b	95.61 ± 0.76^c
300	72.81 ± 5.93^a	85.09 ± 21.43^b	93.86 ± 2.74^c	87.72 ± 1.52^b
200	71.06 ± 2.28^a	93.86 ± 2.01^b	84.21 ± 7.32^c	88.16 ± 2.01^d
100	30.70 ± 5.32^a	86.40 ± 0.76^b	62.72 ± 3.31^c	83.77 ± 0.76^d

Data are presented as means \pm SD of three replicates. Means along a row with different superscripts are significantly different ($p < 0.05$).

The acetone and aqueous extracts' protection against erythrocyte membrane lysis was significantly lower than the standard drug in a dose dependent manner, except for the aqueous extract at 500 µg/ml which were similar in inhibition. The essential oil's protection against erythrocyte membrane induced haemolysis is significantly lower than the standard drug. The oil's activity (IC₅₀ value of 470.2 µg/ml) was 3.6, 3.3, 3.3, 3.6, 3.1 and 3.2 times lower than the standard drug (IC₅₀ value of 482.2 µg/ml) at concentrations 100, 200, 300, 400, 500 and 1000 µg/ml, respectively (Table 1 and Table 3).

The estimated IC₅₀ values indicate that acetone extract and the essential oil exhibited higher protein denaturation inhibitory effect than the aqueous extract while the latter exhibited the highest membrane lysis protection (Table 1).

Discussion

Medicinal plants play important roles as source of antioxidant and anti-inflammatory agents. These bioactivities are mainly due to the presence of secondary metabolites such as polyphenols (Ravipati et al., 2012). This activity could be due to their ability to adsorb, neutralize and reduce free radicals (Mandade et al., 2011). Their ability as free radical scavengers could also be attributed to their redox properties and presence of conjugated ring structures and carboxylic group (Mandade et al., 2011). The mode of action of phenolic compounds in free radical mopng activity is via inactivating lipid free radicals, preventing the decomposition of hydroperoxides into free radicals, chelating metal ions, quenching, up-regulating or protecting antioxidant defenses (Sharma and Singh, 2012). Some act through suppressing the superoxide-driven Fenton reaction which is the most important source of reactive oxygen species. Some antioxidants act through lipid peroxidation inhibition via the inhibition of cyclooxygenase enzyme (Gulcin et al., 2010).

Table 3: Effect of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* on erythrocyte membrane haemolysis inhibitory activity.

Concentration ($\mu\text{g/ml}$)	Acetone extract	Aqueous extract	Essential oil	Diclofenac sodium
1000	70.72 ± 0.77^a	79.50 ± 1.41^b	28.15 ± 0.39^c	89.19 ± 1.78^d
500	56.31 ± 0.78^a	75 ± 1.17^b	25.9 ± 0.38^c	79.95 ± 6.14^b
400	34.69 ± 0.79^a	62.61 ± 2.17^b	22.07 ± 0.78^c	79.51 ± 2.17^d
300	30.63 ± 0.39^a	44.14 ± 0.78^b	18.92 ± 1.17^c	63.06 ± 2.81^d
200	31.08 ± 0.68^a	41.89 ± 1.35^b	17.79 ± 0.39^c	58.56 ± 1.03^d
100	21.4 ± 0.78^a	16.44 ± 0.39^b	16.44 ± 1.56^b	58.55 ± 0.78^c

Data are presented as means \pm SD of three replicates. Means along a row with different superscripts are significantly different ($p < 0.05$).

In the present study, the *in vitro* antioxidant model assays revealed that the plant species possesses free radical scavenging potential according to the free radical present (Figure 1 to 6). The reducing power of essential oil, aqueous and acetone extract of *B. abyssinica* obtained in this study was determined by measuring the conversion of Fe^{+3} to Fe^{+2} . The result obtained showed that the plant fractions possessed antioxidant activity which was dose dependent (Figure 1). This was characterized by the formation of Perl's Prussian blue coloration after ionic reduction, to produce a reduction in the ferric ion/ ferricyanide complex to ferrous form across all the concentration assayed. This activity could be ascribed to electron transfer capability by the metabolites present (Sharma et al., 2012). This suggests that *B. abyssinica* may possess the ability to minimize oxidative damage to some vital tissues in the body and attenuate pathogenesis of disease (Rahman, 2007).

The DPPH molecule is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals (Alam et al., 2013). The delocalization of electron also gives rise to the deep violet color. When a solution of DPPH is mixed with that of a substrate that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour measurable spectrometrically (Alam et al., 2013). The results from a series of concentration ranging from 0.025 to 0.5 mg/ml of the plant extracts and the standards showed different strengths of violet colour during the study. The observed inhibitory potential of the plant fractions against the DPPH radical was dose dependent indicating its potential in relieving oxidative stress (Figure 2).

In biological tissues, nitric oxide synthases metabolizes arginine to citrulline with the generation of nitric oxide radical ($\text{NO}\cdot$) via a five electron oxidative reaction (Boora et al.,

2014). During the *in vitro* studies, the compound sodium nitroprusside decomposes in aqueous solution at physiological pH (7.2) producing NO \cdot , making it an ideal assay to mimic the body system in scavenging the free radical (Pacher et al., 2007). The absence of pro-oxidant in the body leaves it vulnerable to this radical causing diabetes mellitus (Pacher et al., 2007). In this study, the results have showed that aqueous extract and the essential oil of *B. abyssinica* have the capability of mopping up this radical (Figure 3). During this assay, NO \cdot generated from sodium nitroprusside reacts with oxygen to form nitrite. It can be postulated that the plant fractions inhibits nitrite formation by directly competing with oxygen, nitric oxide and other nitrogen oxides such as NO $_3$, N $_2$ O $_4$ and N $_2$ O $_3$ in the reaction (Boora et al., 2014).

Hydrogen peroxide is a highly reactive oxygen species because of its ability to penetrate biological membranes (Chen et al., 2012). It is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH \cdot) that can initiate lipid peroxidation and cause tissue damage (Chen et al., 2012). The scavenging activities of the acetone extract of this plant were 6 fold higher than the reference compounds (Figure 4) indicating the presence of bioactive compounds that could donate electrons to OH \cdot generated in the assay and thus neutralize it to water (Lobo et al., 2010).

The ABTS \cdot^+ radical is a blue chromophore produced by the reaction between ABTS and potassium persulfate (Hazra et al., 2008). In this study, the addition of the plant fractions to this pre-formed radical cation reduced it to ABTS in a concentration-dependent pattern. The potentials were compared to BHT and rutin whose estimated IC $_{50}$ value demonstrates that the acetone extract is a more potent antioxidant (Figure 5).

The inhibitory effect of the *B. abyssinica* fractions on the oxidative cell damage caused by free radicals generated from Fenton mediated reaction was investigated by lipid peroxidation assay. In a biological system, a number of end products of oxidative stress lead to cell membrane obliteration and cell injuries (Moukette et al., 2015). The results of protective activity of the plant fractions against lipid peroxidation indicated that the acetone extract offered significantly the greatest scavenging effects than the other fractions or controls (Figure 6). The inhibition of lipid peroxide by antioxidant compounds is a crucial property by which they can diminish the induction and/or propagation of oxidative stress (Kumar et al., 2013). Thus, this indicates that this species possess a potential propriety as a protective compound against oxidative stress.

Protein denaturation and membrane leakage are the main causes of inflammatory processes implicated in pathogenesis of diseases (Rahman et al., 2012). The mechanism of protein denaturation probably involves alteration of electrostatic, hydrogen, hydrophobic and disulfide bonding in protein conformation (Rahman et al., 2012). In this study, acetone extract and the essential oil exhibited remarkable albumin denaturation inhibitory activity comparable to standard drug indicating protein degradation inhibitory effects (Table 1 and 2).

To confirm the membrane stabilizing activity of *B. abyssinica*, the extracts were tested on the erythrocyte membrane. The red blood cell (RBC) membranes are similar to lysosomal membrane components. Exposure of red blood cell to deleterious substances such as hypotonic medium and toxins results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin (Majumder et al., 2008). The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to the RBC membrane will further render the cell more

susceptible to secondary damage through free radical-induced lipid peroxidation (Majumder et al., 2008). Therefore, the prevention of hypotonicity-induced RBC membrane lysis was taken as a measure of anti-inflammatory activity of plant extracts and the standard drug (Anosike et al., 2012). The results showed that the aqueous extract of this species protected the erythrocyte membrane against hypotonic induced lysis more than the acetone extract and the essential oil (Table 1 and 3). The activity was comparable to that of Diclofenac sodium indicating the species may contain metabolites that could inhibit lysosomal membrane lysis or stabilize the membranes. Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reactions hence preventing the release of phospholipases that trigger the formation of inflammatory mediators (Majumder et al., 2008). These assays allude that the species constituents could possess anti-inflammatory agents.

In conclusion, the present findings demonstrate that the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* possess *in vitro* antioxidant and anti-inflammatory activities. Antioxidant capability may imply to be another mode of action of this plant as an antidiabetic plant via relieving the oxidative stress and attenuating inflammation thus can mediate pathogenesis of diabetes mellitus.

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CHAPTER 5

**ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF THE ESSENTIAL OIL,
ACETONE AND AQUEOUS WHOLE PLANT EXTRACTS OF *BULBINE*
ABYSSINICA AGAINST A SELECTED OPPORTUNISTIC ISOLATES
IMPLICATED IN INFECTIONS ASSOCIATED WITH DIABETES MELLITUS.**

CHAPTER FIVE

Antibacterial and antifungal activities of the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* against a selected opportunistic isolates implicated in infections associated with diabetes mellitus.

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Introduction

Diabetic patients are prone to develop bacterial and fungal infections. These infections include; gastrointestinal, pulmonary, respiratory, urogenital tract, central nervous system, oral cavity, skin, liver, head and neck infections (Holt, 2004). If untreated, these secondary infections cause body failure and even death (Casqueiro et al., 2012).

The bacterial isolates implicated in these infections include *Streptococcus pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Balachandar et al., 2002). *Shigelle flexener*, *Proteus vulgaris*, *Klebsiella pneumonie* and *Enterococcus fercalis* lead to diabetes foot ulcer (Alavi et al., 2007; Gadkowski and Stout, 2008; Al-Salihi and Jumaah, 2013; Appannanavar et al., 2014). The bacteria associated with gastrointestinal and urinary tract infections include *Klebsiella pneumonie*, *Streptococcus pyogens* and *Serratia marcescens* (Hoepelman et al., 2003; Prabuseenivasan et al., 2006; Longdoh et al., 2013).

The fungal isolates associated with these infections are from the *Candida*, *Apergillus*, *Rhizopus*, *Absidia*, *Penicillium*, *Trichophyton* and *Rhodotorula* genus. The *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium aurantiogriesum* and *Penicillium chrysogenum* are associated with oral cavity infections (Drozdowska and Drzewoski, 2008). *Trichophyton rubrum*, *Trichophyton mucoides* and *Trichophyton tonsurans* cause skin and diabetic foot infections. *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichophyton mucoides* and *Trichophyton tonsurans* are associated with gastro-intestinal, pulmonary and central nervous system infections (Chin-Hong, 2006; Eckhard et al., 2007).

These infections are treated using the conventional antibiotics. However, resistance to these drugs by pathogenic microorganisms has increased and this has caused serious clinical problems in the treatment of these infections (Mishra et al., 2007). These drugs are also inaccessible, expensive and have side effects such as bloating, abdominal pain, diarrhea and loss of appetite. These drawbacks have led to seeking alternative therapy from plant remedies (Bastos et al., 2009).

Among these plant remedies, is the genus *Bulbine* (Asphodelaceae) which comprises about forty species in South Africa. In African folk medicine, *Bulbine* species are used in the treatment of sexually transmitted diseases, dysentery, wound and urinary tract infections (Wanjohi et al., 2005). In South Africa, *Bulbine* species are used in the treatment of wounds, burns, rashes, itches, ringworm, cracked lips and herpes (Hutchings et al., 1996).

B. abyssinica is a member of this genus. In Southern African folk medicine, it is used in the treatment of many ailments including cough, vaginal infections, bladder infections and diabetes mellitus (Wanjohi et al., 2005; Oyedemi et al., 2009).

There is scanty literature on the investigations which have been performed on *B. abyssinica* in order to validate its scientific use in folk medicine in South Africa. Studies have only shown that stems and roots of this species possess anti-bacterial properties due to presence of anthraquinones (Van Wyk et al., 1995). However, there is no detailed pharmacological literature on the antibacterial and antifungal potentials of this plant on secondary infections associated with diabetes status.

Therefore, the objective of this study was to evaluate the antibacterial and antifungal activities of the essential oils, acetone and aqueous extracts of this plant using standard procedures in order to relate our findings to their folklore uses in the treatment of infections.

Materials and Methods

Plant materials

The whole plant parts of *B. abyssinica* including the leaves, flowers, stems and roots were collected and authenticated as described in Chapter 3.

Preparation of plant extracts

Some of the plant samples were air-dried, ground to homogeneous powder and extracted using acetone and water. The acetone and aqueous extraction was done as described in Chapter 3. The resulting extracts were reconstituted with their respective solvents to yield a 50 mg/ml stock solution (Otang et al., 2012).

The essential oil was extracted from freshly collected whole plant samples through hydro-distillation method according to the procedure of the European Pharmacopoeia (2002), as described in Chapter 4. The average percentage yield was 0.9%. The essential oil was dissolved with 10% DMSO to yield a 50 mg/ml stock solution for antimicrobial assays.

Microorganisms and media

The bacteria and fungi used in this study were chosen primarily on the basis of their importance as opportunistic pathogens of humans with diabetes mellitus (Longdoh et al., 2013; Appannanavar et al., 2014). The bacterial strains include; *Shigelle flexener* KZN, *Proteus vulgaris*, *Klebsiella pneumonia* ATCC 4352, *Staphylococcus aureus*, *Enterococcus fercalis* ATCC 29212, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* ATCC 19582 and

Serratia marcescens ATCC 9986. The fungi strains of the American Type Culture Collection (ATCC) were used. These include; *Aspergillus fumigatus* ATTC 204305, *Aspergillus niger* ATCC 16888, *Microsporum canis* ATCC 36299, *Microsporum gypseum* ATCC 24102, *Penicillium aurantiogriesum* ATCC 16025, *Penicillium chrysogenum* ATCC 10106, *Trichophyton rubrum* ATCC 28188, *Trichophyton mucoides* ATCC 201382 and *Trichophyton tonsurans* ATCC 28942. The bacteria and fungi test organisms were obtained and purchased from the Department of Biochemistry and Microbiology, University of Fort Hare, South Africa and, Davies Diagnostics (Pty) Ltd, South Africa, respectively.

The Mueller-Hinton dextrose agar (MDA), Mueller-Hinton dextrose broth (MDB), potato dextrose agar (PDA) and sabouraud dextrose broth (SDB) were prepared according to the manufacturer's instructions. The nutrient agar was suspended in demineralized water, boiled while stirring until completely dissolved. It was autoclaved at 121°C for 15 min. The bacteria and fungi were maintained at 4°C on MDA and PDA plates, respectively. The inoculums for the assays were prepared by diluting scraped cell mass in 0.85% sodium chloride solution, adjusted to 0.5 McFarland standards and confirmed by spectrophotometric reading at 580 nm assays (Otang et al., 2012).

The cell suspensions were finally diluted 1:100 in nutrient broth to give an approximate inoculum of 10^4 CFU ml⁻¹ as compared with McFarland standard for use in the assays (Otang et al., 2012).

***In vitro* antibacterial and antifungal activity test**

The agar well diffusion technique was employed as described by Prabuseenivasan et al. (2006) and Otang et al. (2012) with some modifications to test for the antibacterial and

antifungal activity. Briefly, 100 µl of 0.5 McFarland solutions of bacterial or fungal strain cultures in 0.85% sterile distilled water (SDW) was placed over the surface of an agar plate and spread using a sterile inoculation loop. Three wells were cut in each agar plate with a cooled, flamed cork borer of 5 mm diameter, and the agar plugs removed with a sterile needle. A 50 µl of the amoxicillin (0.0125 mg/ml) or nystatin (0.03 mg/ml) were added to the first well for antibacterial and antifungal susceptibility test, respectively to serve as a positive control. In the second well, 50 µl of the corresponding extract solvent were added. In the third well, 50 µl of the acetone extract/aqueous extract/essential oil (50 mg/ml) were added. The essential oil had been prepared by dissolving in 10% aqueous DMSO with Tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through a 0.45 µm membrane filter (Prabuseenivasan et al., 2006). Preliminary studies confirmed that the carrier vehicle did not inhibit the growth of bacteria. Each test was done in triplicate. The culture plates were then incubated at 37°C, and the results were observed after 24 h and 72 h for antibacterial and antifungal susceptibility test, respectively. The clear zone around each well was measured in mm, indicating the activity of the plant fractions against the tested organisms.

Minimum inhibitory concentration (MIC) assay

The broth micro dilution method using 96 well microtitre plates was employed to determine the minimum inhibitory concentration (MIC) of the plant extracts that showed antibacterial or antifungal activity (Otang et al., 2012). Briefly, 120 µl of SDW was added into each well of the first (A) and last (H) rows and also into all the wells of the last column. Then, 120 µl of Nutrient broth (NB) was added into each well of the second row (B). A 150 µl of NB was then added into the remaining wells of the first column and 100 µl into the rest of the wells from the second column rightward. Fifty microliters of the acetone extract/aqueous

extract/essential oil (20 mg/ml) were then added into the third well of the first column while 50 µl of the positive (amoxicillin or nystatin) and negative control (SDW) were separately added into the remaining wells of the first column. A two-fold serial dilution was done by mixing the contents in each well of the first column (starting from the third row) and transferring 100 µl into the second well of the same row and the procedure was repeated up to the 11th well of the same row while discarding 100 µl after the 11th well. Therefore, the dilution of the plant fractions and the control in the wells resulted in a concentration range from 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.019, 0.0098 and 0.005 mg/ml.

Thereafter, 20 µl of 0.5 McFarland bacterial or fungal suspensions was inoculated into the wells except those which contained SDW. The growth of the test organism was measured by determining the absorbance at 620 nm with an automatic ELISA microplate reader (SynergyMx BiotekR, USA) before and after incubation. The plates were incubated at 37°C for 24 h and 72 h for bacteria and fungi, respectively. The MIC 50 was defined and recorded as the concentration of the test antibacterial and antifungal agent that showed 50% inhibition of bacteria and fungi growth, respectively. This was determined by calculating the difference in absorbance between the test wells and the control wells that had the broth and antimicrobial agent alone without the test bacteria or fungi (Penduka et al., 2014). Each test was done in triplicate.

Statistical analysis

The experimental data was expressed as mean \pm standard deviation (SD) of the three replicates. The data was subjected to one way analysis of variance (ANOVA) to compare the difference among the antimicrobial activity of the plant fractions and against the standard

drug using the Minitab program (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). $p < 0.05$ were considered significant.

Results

The results show that the acetone and aqueous extracts were active against the growth of all test organisms except *S. marcescens*. The essential oil was also active against the growth of all test organisms except *K. pneumonia*, *S. pyogens* and *S. marcescens*. The zones of inhibition were varying from 11.7 to 41 mm (Table 1).

The highest activity against the tested bacteria was obtained with the aqueous extract which showed inhibition zones diameters of 41, 19 and 18 mm against *S. aureus*, *P. vulgaris*, *S. flexneri*, respectively. While with acetone extract's inhibitory activity was with inhibition zones diameters of 35, 24, 23 and 20 mm against *S. aureus*, *E. faecalis*, *S. flexneri* and *P. vulgaris*, respectively. The essential oil showed only a significant inhibitory effect against *S. aureus* and the lowest potential against *P. vulgaris* with inhibition diameter of 12 mm. The lowest significant activity obtained with aqueous extract was inhibition zone diameter of 12 mm against *P. aeruginosa*, while with acetone extract, activity against *S. pyogens* showed the lowest inhibition zone diameter of 14 mm. Based on the overall mean inhibition diameters, acetone extract showed significantly more inhibitory activity with overall mean inhibition diameter of 19 ± 9.4 mm than aqueous extract and essential oil with overall mean inhibition diameters of 17.1 ± 10.6 and 11.5 ± 6.6 mm, respectively (Table 1).

Table 1: Inhibition zone diameters caused by the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* (50 mg/ml) against the tested opportunistic bacteria.

Sample	Inhibition zone diameter (mm)							
	Sf	Pa	Sa	Ef	Kp	Sp	Pv	Sm
Acetone extract	23.33 ± 3.06 ₁ ^a	20 ± 3.46 ₁ ^a	35 ± 2.65 ₂ ^a	24 ± 3.46 ₁ ^a	14.67 ± 0.58 ₃ ^a	14.33 ± 1.53 ₃ ^a	20.33 ± 0.58 ₁ ^a	0
Aqueous extract	17.67 ± 6.03 ₁ ^b	12 ± 1.73 ₁ ^b	41 ± 3.46 ₂ ^b	14.67 ± 6.35 ₁ ^b	16 ± 1.73 ₁ ^a	16.67 ± 2.08 ₁ ^a	18.67 ± 1.15 ₁ ^b	0
Essential oil	14.67 ± 1.15 ₁ ^c	16.33 ± 0.58 ₁ ^c	34 ± 5 ₂ ^a	15.33 ± 4.04 ₁ ^b	0	0	11.67 ± 2.89 ₁ ^c	0
*Positive control	22.67 ± 2.31 ₁ ^a	27 ± 3.46 ₁ ^d	30.67 ± 3.51 ₂ ^c	26.67 ± 3.79 ₁ ^a	23.67 ± 0.58 ₁ ^b	38.33 ± 1.53 ₃ ^b	41.67 ± 2.89 ₄ ^d	32.56 ± 2.4 ₂

The bacteria isolates are denoted as Sf (*Shigelle flexneri*), Pa (*Pseudomonas aeruginosa*), Sa (*Staphylococcus aureus*), Ef (*Enterococcus faecalis*), Kp (*Klebsiella pneumonia*), Sp (*Streptococcus pyogenes*), Pv (*Proteus vulgaris*) and Sm (*Serratia marcescens*). Data are presented as means ± SD of three replicates; values along a row with different subscripts are significantly different (P < 0.05). Mean with the different superscript in the same column are significantly different (P < 0.05). *Concentration of positive control (Amoxicillin) is 0.013 mg/ml.

The varying concentrations between 5 and 0.005 mg/ml of the plant fractions were tested in order to determine their MIC 50's. The lowest MIC (0.31 mg/ml) with acetone extract was observed against *S. aureus*, *P. aeruginosa* and *E. faecalis*. The aqueous extract showed the lowest MIC activity against *S. aureus* (0.078 mg/ml) and *S. flexneri* (0.31 mg/ml). The acetone extract (MIC of 1.25 mg/ml) showed significantly similar activity with the standard drug/control (Amoxicillin) against *P. vulgaris*. The lowest MIC was observed with *S. aureus*, *P. aeruginosa* and *E. faecalis* when incubated with the essential oil with the estimated values of 0.16, 0.31 and 0.63 mg/ml, respectively. The essential oil's MIC was significantly higher than the standard drug against all bacteria except against *S. aureus* which exhibited a significantly similar MIC (Table 2).

B. abyssinica was also tested against nine selected diabetic status opportunistic fungi. The results indicated that the plant fractions only inhibited the growth of 33% of the tested organisms. The essential oil, acetone and aqueous extracts were inactive against the growth of all organisms except *M. canis*, *M. gypseum* and *T. rubrum*. The zones of inhibition varied from 19.3 to 33.3 mm. The highest activity was observed with the essential oil which showed inhibition zone diameter of 33 mm against *T. rubrum*. While with acetone extract's inhibitory activity was with inhibition zone diameters of 33 and 29 mm against *M. canis* and *M. gypseum*, respectively. The lowest activity was obtained with aqueous extract showing inhibition zone diameter of 19 mm against *M. gypseum* (Table 3).

The lowest MIC 50 (2.5 mg/ml) observed was against *M. canis* and *M. gypseum* with acetone extract and against *M. gypseum* with aqueous extract. The essential oils showed MIC activity (5 mg/ml) against *T. rubrum*. All the plant fractions showed significantly lower activity when compared to the positive control (nystatin) against all the fungi tested (Table 4).

Table 2: Minimum inhibitory concentrations (MIC) of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* against the tested opportunistic bacteria.

Sample	MIC (mg/ml)							
	Sf	Pa	Sa	Ef	Kp	Sp	Pv	Sm
Acetone extract	0.63 ₁ ^a	0.31 ₂ ^a	0.31 ₂ ^a	0.31 ₂ ^a	2.5 ₃ ^a	> 5 ₄ ^a	1.25 ₅ ^a	-
Aqueous extract	0.31 ₁ ^b	> 5 ₂ ^b	0.078 ₃ ^b	1.25 ₄ ^b	2.5 ₅ ^a	5 ₂ ^a	0.63 ₆ ^b	0
Essential oil	5 ₁ ^c	0.31 ₂ ^a	0.16 ₃ ^c	0.63 ₄ ^c	0	0	5 ₁ ^c	0
*Positive control	0.16 ₁ ^d	0.16 ₁ ^c	0.16 ₂ ^c	0.16 ₁ ^d	0.16 ₁ ^b	0.16 ₃ ^b	1.25 ₄ ^d	0.16

The bacteria isolates are denoted as Sf (*Shigelle flexneri*), Pa (*Pseudomonas aeruginosa*), Sa (*Staphylococcus aureus*), Ef (*Enterococcus faecalis*), Kp (*Klebsiella pneumonia*), Sp (*Streptococcus pyogens*), Pv (*Proteus vulgaris*) and Sm (*Serratia marcescens*). ‘-’ denotes that values not determined. Data are presented as means ± SD of three replicates. Values along a row with different subscripts are significantly different (P < 0.05). Values with the different superscript in the same column are significantly different (P < 0.05). *Concentration of positive control (Amoxicillin) is 0.013 mg/ml.

Table 3: Inhibition zone diameters caused by the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* (50 mg/ml) against the tested opportunistic fungi.

Sample	Inhibition zone diameters (mm)								
	Af	An	Mc	Mg	Pa	Pc	Tr	Tm	Tt
Acetone extract	0	0	32.67 ± 7.50 ₁ ^a	28.67 ± 2.08 ₂ ^a	0	0	0	0	0
Aqueous extract	0	0	0	19.33 ± 3.21 ^b	0	0	0	0	0
Essential Oil	0	0	0	0	0	0	33.33 ± 1.15 ^a	0	0
*Positive control	29 ± 3.61 ₁	32.67 ± 1.15 ₂	31 ± 2 ₂ ^a	23.67 ± 0.58 ₁ ^c	29.67 ± 2.31 ₁	27.33 ± 1.15 ₁	36 ± 8.7 ₃ ^a	35.33 ± 2.31 ₃	30 ± 3.61 ₁

The fungi isolates are denoted as Af (*Aspergillus fumigatus*), An (*Aspergillus niger*), Mc (*Microsporium canis*), Mg (*Microsporium gypseum*), Pa (*Penicillium aurantiogriesum*), Pc (*Penicillium chrysogenum*), Tr (*Trichophyton rubrum*), Tm (*Trichophyton mucoides*) and Tt (*Trichophyton tonsurans*). Data are presented as means ± SD of three replicates; values along a row with different subscripts are significantly different (P < 0.05). Mean with the different superscript in the same column are significantly different (P < 0.05).

*Concentration of positive control (Nystatin) is 0.03 mg/ml.

Table 4: Minimum inhibitory concentrations (MIC) of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* against the tested opportunistic fungi.

Sample	MIC (mg/ml)								
	Af	An	Mc	Mg	Pa	Pc	Tr	Tm	Tt
Acetone extract	-	-	2.5 ₁ ^a	2.5 ₁ ^a	-	-	-	-	-
Aqueous extract	-	-	-	2.5 ^a	-	-	-	-	-
Essential Oil	-	-	-	-	-	-	5 ^a	-	-
*Positive control	-	-	0.312 ₁ ^b	0.625 ₂ ^b	-	-	0.625 ₂ ^b	-	-

The fungi isolates are denoted as Af (*Aspergillus fumigatus*), An (*Aspergillus niger*), Mc (*Microsporium canis*), Mg (*Microsporium gypseum*), Pa (*Penicillium aurantiogriesum*), Pc (*Penicillium chrysogenum*), Tr (*Trichophyton rubrum*), Tm (*Trichophyton mucoides*) and Tt (*Trichophyton tonsurans*). ‘-’ denotes that values not determined. Data are presented as means \pm SD of three replicates. Values along a row with different subscripts are significantly different ($P < 0.05$). Values with the different superscript in the same column are significantly different ($P < 0.05$). *Concentration of positive control (Nystatin) is 0.03 mg/ml.

Discussion

A number of antibiotics are currently available in the management of various infections (Odonkor and Addo, 2011). However, these conventional remedies have continued to be a challenge in clinical control of some selected pathogens causing these diseases. These include the cost and inaccessibility of these antibiotics. This has led to seeking alternative remedies (Olajuyigbe and Afolayan, 2012). Moreover, the increasing capability of microbes to develop multidrug resistance has further encouraged the search for new, safe and effective bioactive agents of plant origin (Olajuyigbe and Afolayan, 2012).

Antimicrobial screening of traditional medicinal plants has been the source of novel antibiotics using different extracts and essential oils (Prabuseenivasan et al., 2006). Over the years, essential oils have shown to be potential sources of novel antimicrobial compounds especially against bacterial pathogens (Njimoh et al., 2015). These plant remedies have enjoyed increasing popularity recently and their therapeutic potentials have continued to be validated using *in vitro* and *in vivo* tests using respective test organisms (Ashafa, 2013). Consequently, many reports have indicated antimicrobial effects of medicinal plants against bacteria and fungi of medical importance (Saad et al., 2011).

In this study, the essential oil, acetone and aqueous whole plant extracts of *B. abyssinica* inhibited the growth of the selected bacteria which are usually implicated in infections associated with diabetes mellitus status. The three plant fractions significantly inhibited the growth of Gram-positive bacteria (*S. flexneri* and *P. aeruginosa*) and Gram-negative bacteria (*S. aureus* and *E. faecalis*). The acetone and aqueous extract also inhibited *K. pneumonia* and *P. vulgaris* (Gram-negative) and *S. pyogenes* (Gram-positive), while essential oil showed activity against *P. vulgaris* (Gram-negative). This implies that this plant possess antibacterial

agents with a broad spectrum activity which could be crucial in the treatment of gastrointestinal and urinary tract infections associated with diabetes mellitus complications (Hoepelman et al., 2003; Prabuseenivasan et al., 2006; Longdoh et al., 2013).

These plant fractions showed a varying degree of activity against the selected bacteria isolates. The difference in antibacterial activity could be attributed to the presence of different chemical constituents due to different extraction solvents used (Lawrence et al., 2009). Different extraction solvents have varying degree of extraction of antibacterial agents (Ramaiya et al., 2014).

Plants are rich sources of bioactive secondary metabolites which could possess antifungal potentials (Arif et al., 2009). In this study, the plant fractions were tested against nine selected opportunistic fungi isolates associated with infections in diabetic status. The results showed that the plant was active against the growth of only three of the tested fungi species. The poor growth inhibitory activity against the selected pathogenic fungi demonstrated by this plant species could be attributed to lack of bioactive antifungal agents extracted using the chosen solvents in this study. Different extraction solvents have varying degree of extraction of metabolic compounds (Lalisan et al., 2014). The fungi resistance towards the metabolites in the plant fractions might also explain the observed poor bioactivity observed with this plant remedy.

The findings in this study indicate that the plant fractions of *B. abyssinica* exhibited a significant antibacterial activity suggesting the presence of good antimicrobial potency. The observed tendency of the fractions to inhibit both Gram-negative and Gram-positive bacteria indicate that this species contain interesting biopharmaceutical substances with ability to

attract significant scientific attention. Though, poor activity was observed with fungi isolates, this plant species could serve as a source of antifungal agents against the susceptible fungi isolates, especially *Microsporum* and *Trichophyton* genera which are known to be resistant to antibiotics and cause deleterious gastrointestinal disorders, central nervous system, skin and foot infections in diabetic patients (Drozdowska and Drzewoski, 2008; Martinez-Rossi et al., 2008).

In conclusion, the extracts with strong antibacterial and mild antifungal activities might produce encouraging clinical outcomes. Notwithstanding, future research is needed to identify the active compounds, appropriate dosages and formulations for clinical use. These findings also scientifically account for the multi-pharmacological use of *B. abyssinica* in the treatment of infections of diabetes mellitus in South Africa traditional medicine.

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CHAPTER 6

**THE EVALUATION OF THE NUTRITIVE VALUE, ELEMENTAL
COMPOSITION, PHYTOCHEMICAL CONSTITUENTS AND COMPOUNDS
RESPONSIBLE FOR THE PHARMACOLOGICAL PROPERTIES OF *BULBINE*
ABYSSINICA.**

CHAPTER SIX

The evaluation of the nutritive value, elemental composition, phytochemical constituents and compounds responsible for the pharmacological properties of *B. abyssinica*.

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Introduction

Medicinal plants have contributed immensely to health care in Africa and are known to play important roles as source of food, therapeutic agents and maintenance of good health. These plants are accessible and cheap sources of important nutrients such as macro and micro elements, vitamins, certain hormone precursors, protein, energy and essential amino acids (Marcel and Bievenu, 2012). The phytoconstituent information is an important aspect in bioprospecting of novel therapeutic agents. Proximate and nutrient analysis of these plant remedies is among the first line in bioprospecting techniques. This analysis plays a crucial role in assessing their nutritional significance and helps to understand the worth of these plants species (Hussain et al., 2009). This is followed by identification of other phytochemicals which form the backbone of the isolation and identification of the crucial therapeutic potentials (Zia-Ul-Haq et al., 2011). These phytoconstituents have nutritional properties and metabolites that are essential in prevention and treatment of chronic diseases such as cancer, cardiovascular disease and diabetes mellitus (Gupta and Prakash, 2009).

Information on the traditional uses of *Bulbine* species in herbal medicine against many diseases have been well documented (Wanjohi et al., 2005, Oyedemi et al., 2009). In addition, some compounds isolated from *B. abyssinica* such as chrysophanol and knipholone, have been found to be active against microbial infections and possess antiplasmodial activity (Bringmann et al., 2002).

In the present study, it has been demonstrated that the plant possesses antidiabetic, antioxidant, anti-inflammatory and antimicrobial properties. Despite this scientific information on *B. abyssinica*, there is no literature available, to the best of my knowledge on

the nutritive value, metabolites or compounds responsible for these observed pharmaceutical properties of this plant in order to justify its use in the treatment of diabetes mellitus and other complications such as infections in Eastern Cape Province. This work therefore was aimed at providing information on the nutritive value and bioactive metabolite(s) of *B. abyssinica* using standard procedures and to relate the findings to their possible functional role in dietary-medicinal uses.

Materials and Methods

Plant materials

The whole plant of *B. abyssinica* including; leaves, flowers, stems and roots were collected and authenticated as described in Chapter 3. One set of fresh samples was kept in the refrigerator at 4°C until time for essential oil extraction as described in Chapter 4. The other samples were properly washed, air dried, ground to fine powder and stored in airtight bottles which were then kept in the refrigerator at 4°C. The pulverized samples were used for nutritive and elemental composition analysis, while others were used for acetone and aqueous extraction as described in Chapter 3.

Chemicals and reagents used

The chemicals used include; Glacial acetic acid (CH_3COOH), Hydrochloric acid (HCL), Sulphuric acid (H_2SO_4), Salicylic acid ($\text{C}_7\text{H}_6\text{O}_3$), Trichloroacetic acid (TCA), Ferric chloride (FeCl_2), Sodium carbonate (Na_2CO_3), Aluminium chloride (AlCl_3), Calcium chloride (CaCl_2), Chloroform (CHCl_3), Acetic anhydride (CH_3CO)₂O, Diethyl ether (C_2H_5)₂O, Sodium hydroxide (NaOH), Ethanol ($\text{C}_2\text{H}_6\text{O}$), Ammonia hydroxide (NH_4OH), Potassium permanganate (KMnO_4), Ammonium thiocyanate (NH_4SCN), α - α -dipyridine ($\text{C}_{10}\text{H}_8\text{N}_2$), Ascorbic acid (Vitamin C) ($\text{C}_6\text{H}_8\text{O}_6$), Vitamin A ($\text{C}_{20}\text{H}_{30}\text{O}$) and Vitamin E ($\text{C}_{29}\text{H}_{50}\text{O}_2$) and

Dimethyl sulfoxide (DMSO). These chemicals were purchased from Merck and Sigma-Aldrich, Gauteng, South Africa. All the chemicals used in this study were of analytical grade.

Nutritive composition analysis

Proximate analysis

The moisture content was determined as described by Bvenura and Afolayan (2012). Briefly, a clean crucible was dried to a constant weight in air oven at 110°C, cooled in a desiccator and weighed (W1). Two grams of finely ground sample was accurately weighed into the previously labeled crucible and reweighed (W2). The crucible containing the sample was dried in an oven to constant weight (W3). The percentage (%) moisture content was calculated as:

$$\text{Moisture content (\%)} = (\text{final weight of the sample after incineration (g)} / \text{weight of initial sample (g)}) \times 100.$$

The ash content of the plant was determined using the method of Antia et al. (2006). Briefly, 5 g of the powdered sample was incinerated in an E-Range muffle furnace with TOHO P4 programme at 550°C for 12 h. The final weight of the sample was used to calculate the ash content as follows:

$$\text{Ash content (\%)} = (\text{final weight of the sample after incineration (g)} / 5 \text{ g}) \times 100.$$

Crude fat was determined as described by Antia et al. (2006). About 5 g of the powdered sample was weighed; 100 ml of diethyl ether was added, covered with aluminium foil and shaken in an orbital shaker for 24 h. It was then filtered and the supernatant decanted. Another 100 ml of diethyl ether was added to the residue and shaken for another 24 h. The residue obtained after filtration was the fat free sample and it was as calculated as follows:

Crude fat = Weight of sample after diethyl ether extraction / Initial weight of sample x 100.

The crude fibre content of the plant was determined also by the method of Antia et al. (2006). Briefly, 5 g of the powdered sample was weighed and digested in 100 ml of 1.25% sulphuric acid for 30 min. The acid digested sample was allowed to cool, and then filtered. The residue was collected for further digestion with 100 ml of 1.25% sodium hydroxide. The sample was then filtered and the residue dried in an oven at 100°C to a constant weight. The dried residue was incinerated in a muffle furnace for 24 h at 550°C. The crude fibre was obtained from the loss in weight on ignition of dried residue remaining after digestion of fat free samples as:

$$\% \text{ fibre} = \text{Loss of weight on ignition} / \text{Weight of sample used} \times 100.$$

The nitrogen content of the plant was determined using the method of Bvenura and Afolayan (2012) by means of the Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). The value of nitrogen obtained was multiplied by 6.25 to give the % crude protein (Magomya et al., 2014).

The carbohydrate content was determined by subtracting the total crude protein, crude fibre, ash and lipid from the total dry matter (Hussain et al., 2013). The caloric value estimation was done by summing the multiplied values for crude protein, crude lipid (excluding crude fibre) and carbohydrate, respectively at Atwater factors (17 kJ, 37 kJ and 17 kJ) (Moses and Yemisi, 2010).

The percentage contribution to energy due to protein (PEP), due to total fat (PEF) and due to carbohydrate (PEC) as PEP%, PEF% and PEC% respectively were calculated. The percentage utilizable energy due to protein (UEDP%) was also calculated according to the method of Jonathan and Funmilola (2014). All the analyses were done in triplicate.

Macro and micro-minerals analysis

Digestion and mineral analysis

The method described by Bvenura and Afolayan (2012) was used for the digestion of plant material. Briefly, a digestion mixture comprising of selenium powder, sulphuric acid and salicylic acid were prepared. About 0.3 g of the ground plant material was placed in dry, clean digestion tubes. A volume of 2.5 ml of the digestion mixture was added to each tube and allowed to react at room temperature for 2 h. The tubes were heated in a block digester at 110 °C for 60 min. The tubes were allowed to cool and three successive portions of 1 ml hydrogen peroxide added at 10 sec intervals due to the volatility of the reaction. The tubes were returned to the block digester at a temperature of 330°C and were removed from the block digester when the digest turned from coloured to clear. The tubes were allowed to cool to room temperature, contents transferred into 50 ml volumetric flasks and then deionized water was added to attain volumes of 50 ml. Standards were prepared for all the individual elements to be analyzed.

The macro-minerals (Calcium, Magnesium, Potassium, Sodium and Phosphorus) and micro-minerals (Iron, Zinc, Aluminium, Manganese and Copper) were determined using the Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES; Varian 710-ES series, SMM Instruments, Cape Town, South Africa) (Bvenura and Afolayan, 2012). All analysis was carried out in triplicates.

Vitamin analysis

The Vitamin C (Ascorbic acid) content of the plant was determined by a modified spectrophotometric method as described by Tahirovic et al. (2012). Briefly, 2.5 g of coarsely powdered sample was weighed and 12 ml of glacial acetic acid added. The mixture was stirred for about 20 min and filtered. The filtered solution was made up to 100 ml using distilled water. From this, 50 ml of the sample solution was mixed with 10 μ l of methylene blue solution (0.4 mmol/l) and diluted to 10 ml with distilled water. Absorption was measured at 665 nm using a spectrophotometer (AJ-IC03).

Stock solution of ascorbic acid (1M) was prepared by dissolving 10 g of ascorbic acid in 56.76 ml of distilled water. The different concentrations were prepared by diluting the stock standard solution in water before use and absorption was also measured at 665 nm. The calibration graph was drawn by plotting the absorbance against concentration of ascorbic acid. The calibration curve obtained was linear in a concentration range of 0.1 to 1M with the linear regression equation as;

$$y = 0.0169x, R^2 = 0.8208, \text{ where } y \text{ is the absorbance and } x \text{ is the concentration of Vit C.}$$

Quantitative determination of Vitamin A was done using the method of Onyesife et al. (2014). Briefly, 1 g of ground sample was macerated with 20 ml of petroleum ether. This was decanted into a test tube and then evaporated to dryness. About 0.2 ml of chloroform-acetic anhydride (1:1 v/v) was added to the residue. A 2 ml of Trichloroacetic acid-chloroform (1:1 v/v) was added to the resulting solution and absorbance was measured at 620 nm. Vitamin A standard was prepared in the same manner and the absorbance at 620 nm recorded. The

concentration of vitamin A in the sample was extrapolated from the standard curve using the calibration curve equation;

$$y = 0.7391x, R^2 = 0.877, \text{ where } y \text{ is the absorbance and } x \text{ is the concentration of Vit A.}$$

The Vitamin E content was determined using the method of Onyesife et al. (2014). Briefly, 1 g of the sample was macerated with 20 ml of ethanol and then filtered. Then, 0.2% ferric chloride in ethanol and 1 ml of 0.5% α - α -dipyridine was prepared and added to 1 ml of the filtrate. This was diluted to 5 ml with distilled water. Absorbance was read at 520 nm. The standard solutions were prepared similarly and the concentration of vitamin E extrapolated from the standard curve using the calibration curve equation;

$$y = 1.5552x, R^2 = 0.824, \text{ where } y \text{ is the absorbance and } x \text{ is the concentration of Vit E.}$$

All the experiments were done in triplicates.

Anti-nutrient analysis

Oxalate was determined by using the method of Naik et al. (2014). Briefly, 1 g of the sample was placed in 250 ml volumetric flask, 190 ml of distilled water and 10 ml of 6 M hydrochloric acid were added. The mixture was then warmed in a water bath at 90°C for 4 h and the digested sample centrifuged at 2,000 rpm for 5 min. The supernatant was then diluted to 250 ml. Three 50 ml aliquots of the supernatant was evaporated to 25 ml, the brown precipitate was filtered and washed. The combined solution was then titrated with concentrated ammonia solution in drops until the pink colour of methyl orange changed to yellow. The solution was then heated in a water bath to 90°C and the oxalate was precipitated with 5% calcium chloride solution which was allowed to stand overnight. This was centrifuged; the precipitate washed with hot 25% sulfuric acid, diluted to 125 ml with

distilled water and titrated against 0.05M potassium permanganate (KMnO₄). Oxalate content was calculated using the formula;

$$1 \text{ ml } 0.05 \text{ M KMnO}_4 = 2.2 \text{ mg Oxalate}$$

Phytic acid was determined by the method of Abidemi (2013). Briefly, 2 g of the sample was weighed into a 250 ml conical flask, soaked in 100 ml of 2% concentrated hydrochloric acid for 3 h and then filtered with a Whatman No. 1 filter paper. To the 50 ml of the filtrate, 10 ml of distilled water were added to give proper acidity. Then, to this solution, 10 ml of 0.3% ammonium thiocyanate solution was added and titrated with standard Iron (II) Chloride solution containing 0.00195 g Iron/ml, end point observed to be yellow which persisted for 5 min. The percentage phytic acid was calculated thus:

$$\% \text{ Phytic acid} = y \times 1.19 \times 100; \text{ Where } y = \text{titre value} \times 0.00195 \text{ g.}$$

The phytonutrient, vitamins, macro and micro element contents were expressed as mg/100g.

Essential oil chemical analysis

Preparation of the essential oil

The essential oil was extracted from the fresh whole plant samples using the hydro distillation method as described in Chapter 4.

Gas chromatography mass spectrometry (GC-MS)

GC-MS analyses were performed on Agilent 5977A MSD and 7890B GC System, Chemetrix (pty) Ltd; Agilent Technologies, DE (Germany) with a Zebron-5MS column (ZB-5MS 30 m x 0.25 mm x 0.25 um) (5%-phenylmethylpolysiloxane). The following column and temperature conditions were used: GC grade helium at a flow rate of 2 ml/min and splitless 1 ml injections were used. The injector, source and oven temperatures were set at 280°C,

280°C and 70°C, respectively. The ramp settings were; 15°C/min to 120°C, then 10°C/min to 180°C, then 20°C/ min to 270°C and held for 3 min.

Identification of compounds

The identification of the chemical constituents of the essential oil was determined by their GC retention times, percentage composition (area %) and retention indices. The Kovat indices were calculated with respect to a set of standard hydrocarbons (C₉-C₂₀) (El-Shazly et al., 2002). The interpretation and identification of their mass spectra was confirmed by mass spectral incorporated library. The identification was further confirmed by search using the National Institute of Standards and Technology (NIST) database (NIST/EPA/NIH mass spectral library 2014) with those of published data (Shahat et al., 2011). Empirical searches were conducted using The PubChem Project (<https://pubchem.ncbi.nlm.nih.gov/>) and DrugBank (www.drugbank.ca/) to identify the known pharmacological properties associated with these compounds.

Phytochemical content analysis of the acetone and aqueous whole plant extracts

Preparation of extracts

The plant samples were properly washed, air dried, pulverized into powder and stored in airtight bottles which were then kept in the refrigerator at 4°C. The acetone and aqueous extracts were prepared as described in Chapter 2.

Determination of total phenols

The total phenolic content in the extracts were determined by the modified Folin-Ciocalteu method of Bouaziz-Ketata et al. (2015). An aliquot of 0.5 ml of each plant extract (1 mg/ml) was mixed with 5 ml of Folin-Ciocalteu reagent which was previously diluted with distilled

water (1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The mixtures were vortexed for 15 sec and allowed to stand for 30 min at 40°C to develop colour. Absorbance was then read at 765 nm using the AJI-C03 UV-VIS spectrophotometer. The results were expressed as mg/g tannic acid equivalent using the equation based on the calibration curve:

$Y = 0.1216 x$; $R^2 = 0.9365$, where x is the absorbance and Y is the tannic acid equivalent.

Determination of total flavonoids

The flavonoid content was determined by the method used by Oyedemi et al. (2010). Briefly, 0.5 ml of 2% $AlCl_3$ was prepared in ethanol. This was then added to 0.5 ml of the extracts. The mixture was allowed to stand for 60 min at room temperature and the absorbance was read at 420 nm. The extracts were evaluated at a final concentration of 0.1 mg/ml. The results were calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve:

$Y = 0.0255 x$; $R^2 = 0.9812$; where x is the absorbance and y is the quercetin equivalent.

Determination of total flavonols

The flavonol content was determined based on the method used by Oyedemi et al. (2010). Briefly, 2 ml of each plant extract were mixed with 2 ml of 2% $AlCl_3$ prepared in ethanol. Then 3 ml of sodium acetate solution (50 g/l) was added. The mixture was incubated at 20°C for 2.5 h. Absorbance was measured at 440 nm. The total flavonol content was calculated as quercetin (mg/g) using the following equation based on the calibration curve:

$Y=0.0255x$; $R^2 = 0.9812$, where x is the absorbance and Y is the quercetin equivalent.

Determination of proanthocyanidin

The total proanthocyanidin was determined using the method described by Oyedemi et al. (2010). A volume of 0.5 ml of the extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml HCL. The resulting mixture was vortexed, allowed to stand for 15 min at room temperature and absorbance read at 500 nm. Total proanthocyanidin content was expressed as catechin equivalents (mg/g) using the calibration curve equation:

$Y = 0.5825x; R^2 = 0.9277$, where x is the absorbance and Y is the catechin equivalent.

Determination of tannins

Tannin determination was done according to the procedure of Mbaebie et al. (2012) with some modifications. A 0.2 g of plant extract was added to 20 ml of 50% methanol. This was mixed thoroughly and placed in a water bath at 80°C for 60 min. The extract was filtered into a 100 ml volumetric flask; 20 ml of distilled water was added, followed by 2.5 ml of Folin-Ciocalteu reagent and 10 ml of 17% Na₂CO₃. This was thoroughly mixed together and made up to 100 ml using distilled water. The mixture was allowed to stand for 20 min until the bluish-green color developed. The different tannic acid standard solutions concentrations used ranged from 0 to 10 ppm. The absorbance of the tannic acid standard solutions and plant extracts were measured after color development at 760 nm using the AJI-C03 UV-VIS spectrophotometer. The results were expressed as mg/g of tannic acid equivalent using the calibration curve:

$Y = 0.0763x; R^2 = 0.9644$, where x is the absorbance and Y is tannic acid equivalent.

Determination of alkaloids

The alkaloid content was determined according to the method of Omoruyi et al. (2012). Briefly, 5 g of plant extract was mixed with 200 ml of 10% acetic acid in ethanol. The mixture was covered and allowed to stand for 4 h. This was filtered and the filtrate was concentrated on a water bath to one-fourth of its original volume. Concentrated ammonium hydroxide was added in drops to the extract until precipitation was completed. The whole solution was allowed to settle, the collected precipitates washed with dilute ammonium hydroxide and then filtered. The residue collected was dried and weighed. The alkaloid content was determined using this formula:

$$\% \text{ alkaloid} = \text{final weight of sample} / \text{initial weight of extract} \times 100.$$

Determination of saponins

The saponin content in the plant extracts was determined using the method of Omoruyi et al. (2012). Briefly, 20 g of the plant extract was mixed with 200 ml of 20% ethanol in a shaker for 30 min and heated in a water bath at 55°C for 4 h with continuous stirring. The mixture was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over the water bath at 90°C. The concentrated solution obtained was then transferred into a 250 ml separating funnel and extracted twice using 20 ml diethyl ether. The ether layer was discarded, while the aqueous layer was retained and 60 ml *n*-butanol was added. The *n*-butanol extracts were washed twice with 10 ml of 5% sodium chloride. The remaining solution was heated in a water bath to evaporate and the samples were oven dried at 40°C to a constant weight. The percentage saponin content was calculated using the formula:

$$\% \text{ saponin} = \text{final weight of sample} / \text{initial weight of sample} \times 100.$$

All the experiments were done in triplicates.

Statistical analysis

All experiments were done in triplicates and the results were expressed as Mean \pm SD. Where applicable, the data was subjected to student t-test to compare the difference between the acetone and aqueous extracts using the Minitab program (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). $p < 0.05$ were considered significant.

Results

The proximate constituents of *B. abyssinica* that were tested include; moisture, ash, crude fibre, carbohydrate, protein and lipids. Out of these, crude carbohydrate was the highest proximate factor. There were relatively similar quantities of moisture, ash, crude fibre and crude protein content in this species. The crude lipids had the lowest concentration (Table 1).

Table 1: The percentage proximate composition of the *B. abyssinica* A. Rich.

Parameters	% Dry matter
Moisture content	8.81 \pm 0.4
Ash content	8 \pm 0.61
Crude fibre	8.85 \pm 0.02
Crude carbohydrate	74.83 \pm 0.64
Crude protein	7.68 \pm 0.22
Crude lipids	0.64 \pm 0.01

Data are presented as means \pm SD of three replicates.

The estimated energy values obtainable from crude protein, carbohydrate, lipid content are represented in Table 2 with total energy due to carbohydrate and lipid being the highest and lowest, respectively. The plant showed higher levels of Vitamin E than Vitamin A and C which showed similar levels (Table 3).

Table 2: Calculated energy values as contributed by protein, fat and carbohydrate in *B. abyssinica* A. Rich.

Parameters	Value
Total energy (kJ/100g)	1426.23 ± 10.82
PEP (%)	9.15 ± 0.27
PEC (%)	89.19 ± 0.27
PEL (%)	1.66 ± 0.01
UEDP (%)	0.39 ± 0.01

PEP%, PEC%, PEF% and UEDP% refers to percentage proportion of total energy due to protein, carbohydrate, fat and utilizable energy due to protein, respectively. Data expressed as mean ± SD of three replicates.

Table 3: Phytonutrient and vitamin content (mg/100g) of *B. abyssinica* A. Rich.

Phytonutrient	Composition (mg/100g)
Vitamins A	12 ± 1.09
Vitamin C	12.33 ± 1.15
Vitamin E	22.05 ± 4.11
Phytic acid	30.94 ± 6.70
Oxalate	39.52 ± 2.07

Data expressed as mean ± SD of three replicates.

The macro-minerals that were tested included; sodium, calcium, potassium, magnesium and phosphorus. Of these, potassium had the highest concentration in the plant. This was followed by calcium, magnesium, sodium and phosphorous, respectively. The micro-minerals that were evaluated included; iron, aluminium, zinc, manganese and copper. Among these minerals, iron had the highest concentration in the plant, followed by aluminium, manganese, zinc and copper, respectively (Table 4).

Table 4: Macro- and micro-element composition (mg/100g) of *B. abyssinica* A. Rich.

Mineral elements	Composition (mg/100g)
Nitrogen	1228.36 ± 35.72
Phosphorus	218.39 ± 0.005
Calcium	1872.21 ± 52.52
Magnesium	310.44 ± 8.94
Potassium	2485 ± 98.29
Sodium	265.13 ± 13.93
Iron	290.85 ± 39.99
Zinc	5.77 ± 0.06
Aluminum	219.24 ± 14.95
Manganese	10.71 ± 1.19
Copper	0.934 ± 0.06

Data expressed as mean ± SD of three replicates.

The phytochemical contents were also evaluated using standard protocols. The results showed remarkable amounts of total phenols, flavonoids, flavanols and proanthocyanidins in acetone and aqueous whole plant extracts of *B. abyssinica*. The alkaloids and saponins content were in small amounts in both extracts while tannins were in trace amounts. The aqueous extract showed significantly higher amounts of all the phytochemical constituents present than the acetone extract except, total flavanols and saponin contents (Figure 1).

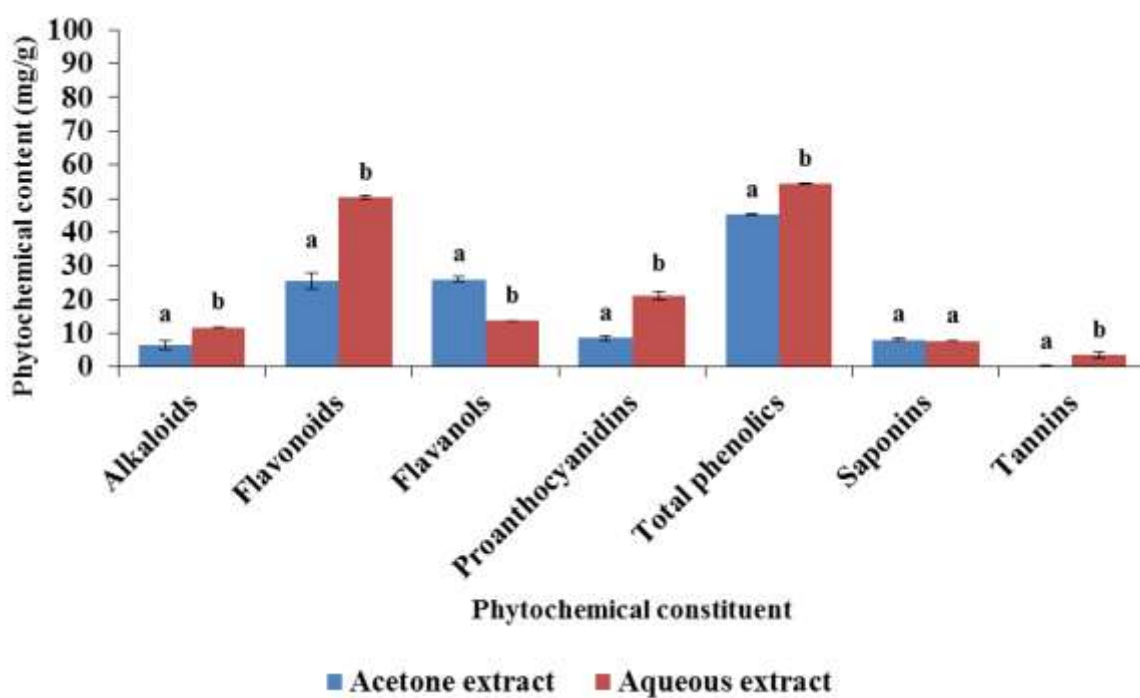


Figure 1: Phytochemical constituents in the acetone and aqueous extracts of *B. abyssinica*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same constituent are significantly different ($P < 0.05$).

The results obtained by GC-MS analysis of the essential oil fraction revealed the presence of 16 compounds. This fraction was characterized by large amounts of terpenes which constituted 91.9 % of the total compounds. Terpinen-4-ol with a MW of 154.2 and a KI of 1041, was the major component (43.68%). Other terpenes present in their decreasing order of quantity are γ -Terpinene, α -Terpinolene, Cymene, 2-Carene and Eucalyptol. A class of esters was also detected and represented 8.09% of the total fraction. The major ester compound was a Phthalic acid, di (oct-3-yl) ester (3.07%) with the MW of 390.6 and RT of 13.281 (Table 5 and Figure 2). Other esteric compounds present in this fraction in their decreasing order of quantity are Di-n-decylsulfone, Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-, 2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-trans-, and Eicosane, 9-octyl- This plant possesses unidentified compounds with unknown biological functions which were observed in the analysis, NIST 2014 library and other literature search. These include all the esteric compounds (Table 5).

Table 5: Chemical composition of the essential oils of the whole plant part of *B. abyssinica*.

S/N	RT	KI	Name	Nature	Molecular formula	Area (%)	Known pharmacological activity	References
1	4.011	943	α -Pinene	Monoterpene	C ₁₀ H ₁₆	1.32	Antifungal and antibacterial	Rivas da Silva et al., 2012
2	4.319	958	β -Phellandrene	Cyclic monoterpene	C ₁₀ H ₁₆	1.27	Antibacterial	Buitragoa et al., 2012
3	4.664	975	α -Terpinolene	Monoterpene	C ₁₀ H ₁₆	7.90	Antioxidant	Aydin et al., 2013
4	4.725	978	Cymene	Monoterpene	C ₁₀ H ₁₄	3.75	Antibacterial	Kisko and Roller, 2005
5	4.805	982	Eucalyptol	Monoterpene alcohol	C ₁₀ H ₁₈ O	3.38	Antioxidant and antibacterial	Zengin and Baysal, 2014
6	4.997	992	γ -Terpinene	Cyclic monoterpene	C ₁₀ H ₁₆	17.49	Antioxidant, anti-inflammatory, antifungal, antibacterial	Foti and Ingold, 2003; Nicholson and Gobert, 2006; Mondello et al., 2006; Oyedemi et al., 2009.
7	5.248	1004	2-Carene	Bicyclic monoterpene	C ₁₀ H ₁₆	3.54	Antioxidant	Emamia et al., 2011
8	5.524	1018	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, trans-	Ester	C ₁₀ H ₁₈ O	1.14	Unknown	-

9	5.977	1041	Terpinen-4-ol	Monoterpene alcohol	C ₁₀ H ₁₈ O	43.68	Anti-inflammatory, antibacterial	Hart et al., 2000; Mondello and Ricci, 2012
10	6.062	1045	α-Terpineol	Monoterpene alcohol	C ₁₀ H ₁₈ O	3.31	Antioxidant, Anti-inflammatory, antibacterial and antifungal	Pitarokili et al., 2002; Brand et al., 2001; Held et al., 2007; Oyedemi et al., 2009.
11	8.263	1127	γ-Elemene	Sesquiterpene	C ₁₅ H ₂₄	2.28	Antioxidant, antifungal	Akpuaka et al., 2013
12	8.837	1152	(-)-Globulol	Sesquiterpene alcohol	C ₁₅ H ₂₆ O	1.26	Antifungal	Tan et al., 2008
13	9.059	1162	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	Ester	C ₁₅ H ₂₄	1.19	Unknown	-
14	13.015	-	Eicosane, 9-octyl-	Ester	C ₂₈ H ₅₈	0.95	Unknown	-
15	13.281	-	Phthalic acid, di(oct-3-yl) ester	Ester	C ₂₄ H ₃₈ O ₄	3.07	Unknown	-
16	13.8	1348	Di-n-decylsulfone	Ester	C ₂₀ H ₄₂ O ₂ S	1.5	Unknown	-

The name, nature, retention time (RT), Kovats index (KI), molecular formula, percentage composition (%) and the known pharmacological properties of the identified compounds in essential oil. KI calculated with respect to a set of standard hydrocarbons (C₉ to C₂₀). “-“means values and pharmacological activity not determined and unknown, respectively.

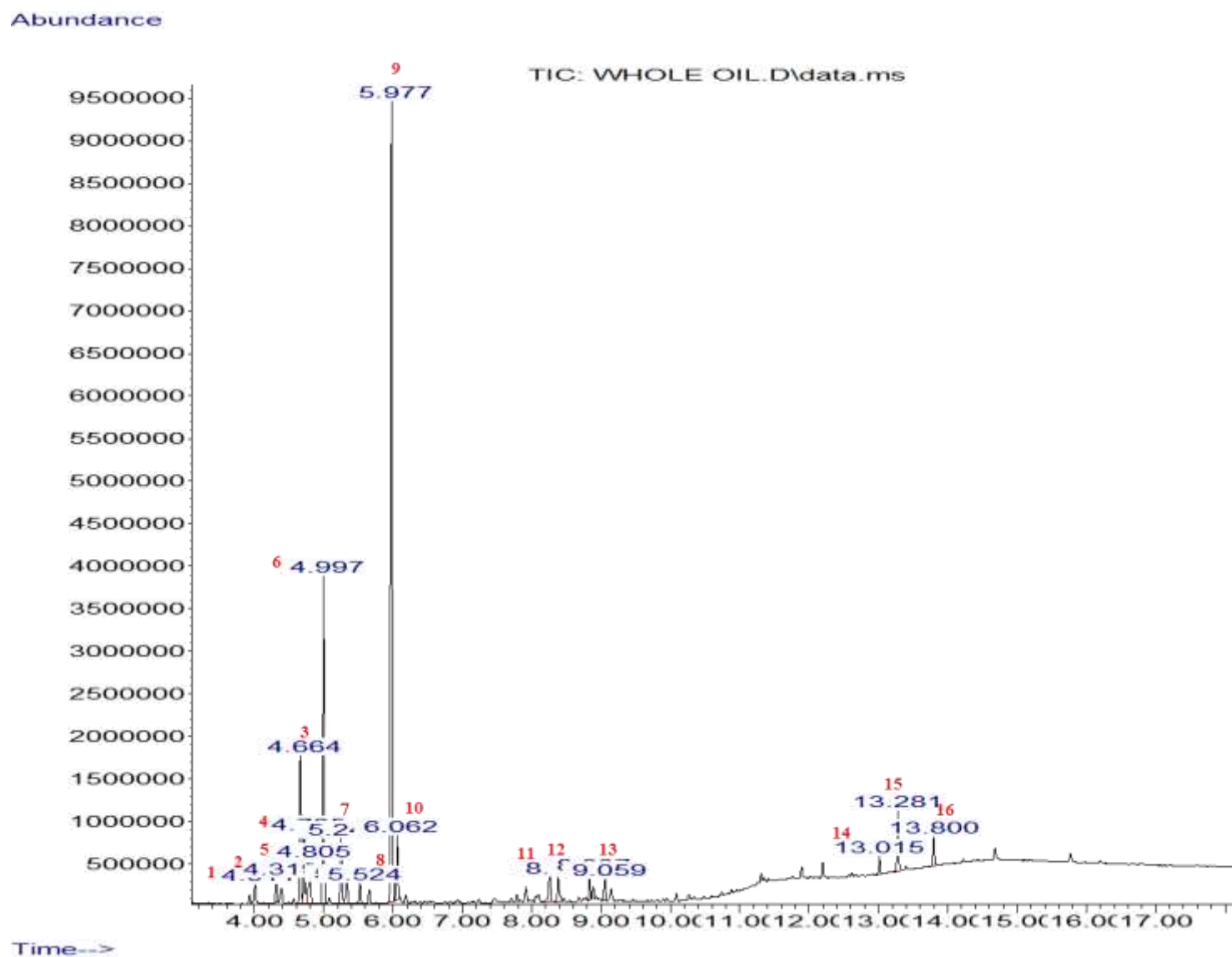


Figure 2: GC-MS chromatogram for essential oil of *B. abyssinica*. Numbers in blue and red colours represents the compound's retention time and their corresponding identity in Table 5, respectively.

Discussion

The presence of crude fiber, moisture, total ash, total protein, lipids and soluble carbohydrate in *B. abyssinica* powder is an indication of the holistic nature of the herbal medicine. Moisture content is important to a number of biochemical reactions, physiological changes and control of body temperature (Igwe and Okwu, 2013). Ash content is a reflection of the mineral contents preserved in the plants. Minerals are essential for the proper functioning of tissues and act as second messengers in some biochemical cascade mechanisms (Antia et al., 2006).

Carbohydrates provide readily accessible fuel for physical performance and regulate nerve tissue (Hussain et al., 2013). The presence of crude fibre is an indicator of the presence of a proportion of cellulose, hemicellulose and lignin (Igwe and Okwu, 2013). Dietary fibre decreases the absorption of cholesterol from the gut, delays the digestion and conversion of starch to simple sugars. Therefore, it lowers the serum cholesterol, the risk of coronary heart disease, hypertension, constipation, diabetes, colorectal cancer and breast cancer (Igwe and Okwu, 2013). Fibers are necessary for digestion and for effective elimination of wastes (Simee, 2011). Thus, this medicinal plant can be considered as a valuable source of dietary fiber in human nutrition (Simee, 2011).

Protein is essential in human system because it functions in the growth, movement and body defense system. Proteins serve as enzymatic catalyst and mediate metabolic and energy regulation (Hussain et al., 2013). The lowest parameter noted was crude fat content (Table 1). Lipids (fats) are major structural elements of biological membranes and in transportation of proteins and vitamins. Lipids in combination with certain proteins (apoproteins) mediate a number of enzyme activities. Lipids also play significant role in the body metabolism as high energy yielding elements and wound healing processes (Igwe and Okwu, 2013).

The total energy provided by carbohydrates from this species is 89.2% more than proteins and fats. The total energy is 1,426.23 kJ/100g while utilizable energy due to protein is 0.39% (Table 2). These two energy values are below the daily energy requirement for an adult and infant (depending on physiological state) as reported by Adeyeye (2014). The PEC% of 89.2% is above the recommended daily energy minimum requirements (45-65%) for human indicating that this plant is valuable source of energy. Energy is important for general health and performance of the body (Hussain et al., 2013).

Vitamin E had the highest concentration while Vitamin A and C had similar concentrations (Table 3). The presence of Vitamin E and C could explain the antioxidant potential noted with this plant as observed in Chapter 4. Vitamin E is the most effective, fat-soluble antioxidant known to occur in the human body. It maintains the integrity of the body's intracellular membranes and provides a defense line against tissue damage caused by oxidation (Ulatowski and Manor, 2013). Vitamin C plays significant functions in the body including; wound healing, activation of enzymes and hormones, antioxidant and strengthening immune system (Iqbal et al., 2004). Vitamin A plays important role in vision, bone growth, reproduction, cell division and differentiation (Martini et al., 2010). These vitamins maintain proper health status of the body. Vitamin A, C and E contents of the plant are sufficient to meet the RDA of the body whose values are 0.3-0.9 mg/day, 30-60 mg/day and 70-300 mg/day in children and adult, respectively (NHMRC, 2005).

Several macro minerals with different concentrations were observed in the plant (Table 4). Potassium (K) is the main intracellular cation in the human body required for vital cellular processes. It is involved in regulating acid–base balance, blood pressure, cell membrane function and basic cellular enzymatic reaction (Chatterjee et al., 2011). The K content (2,485 mg/100g) is

within the RDA of 2,400-3,800 mg/day in children and adults, respectively, hence the species is a good source of the mineral (NHMRC, 2005).

Calcium (Ca) is a mineral needed for optimal bone health and physiological functioning. Calcium acts as a vital second messenger in blood coagulation, hormone secretion action, muscle contraction and nerve function (Pravina et al., 2013). This plant can act as a source of Ca. It contains Ca content (1,872.2 mg/100g) which is merely within the RDA of 500-1,300 mg/day in children and adults, respectively (FAO, 2001).

The plant provides magnesium (Mg) content (310.4 mg/100g) which is below the RDA of 450 mg/day in human (Abrams et al., 1997). The presence of Mg in this plant suggests the antidiabetic potential of this species. Mg acts as a cofactor to several enzymes (like kinases) which participate in energy and protein production processes. It's also vital in strengthening cell membrane structure and modulates glucose transport across cell membranes (Jahnen-Dechent and Ketteler, 2012). Studies have shown that Mg supplementation improves insulin sensitivity in diabetic patients and it can improve insulin sensitivity in obese individuals who are at risk of type 2 diabetes mellitus (Volpe, 2013).

The plant contains sodium (Na) content (265.1 mg/100g) which is below the RDA of 460-920 mg/day in children and adults, respectively (NHMRC, 2005). However, its trace level is still important. Na is the principal cation in extracellular fluids. It maintains the osmotic pressure of the body fluids and preserves normal function of the nervous and muscular system (Constantin and Alexandru, 2011). The plant provides phosphorus (P) (218.4 mg/100g) which is within the RDA of 200-1,000 mg/day in children and adults, respectively (FNB, 1997). Phosphorus is located in every cell of the body and functions as a constituent of bones, teeth, phosphorylated

metabolic intermediates and nucleic acids. It is involved in synthesis of phospholipids and phosphor-proteins (Soetan et al., 2010).

Some micro-minerals were also detected in this plant (Table 4). The species contains iron (Fe) content (290.9 mg/100g) which is above the RDA of 9-15 mg/day in children and adults, respectively (NHMRC, 2005). Fe is an important element; it helps in transport of oxygen, electron transport and blood formation. Fe is crucial in energy production, neurotransmitter synthesis and maintaining a stable immune system (Linder, 2013). Fe plays a key role in relieving oxidative stress in diabetic status thereby decreasing internalization of insulin to its receptors (Bertelsen et al., 2001). Fe down-regulates genes such as hepcidin, LXR α and FPN which are key immunological factors hence reduce cellular reactive oxygen species (ROS), tissue damage, lipid retention and inflammation. This implies iron's role as a therapeutic agent against inflammation and atherosclerosis conditions which are associated with diabetes mellitus (Habib and Finn, 2014). The presence of this mineral in this herbal remedy and its folklore use could explain the observed antidiabetic, antioxidant and anti-inflammatory activities seen in Chapter 3 and 4, respectively.

The second highest micro mineral in the plant was aluminium (Al). The presence of Al is a cause for concern, taking into account that this micro mineral is toxic when consumed in large quantities (Watanabe and Osaki, 2002). The species provides manganese (Mn) content of 10.7 mg/100g. This is slightly above the RDA of 2-5 mg/day in children and adults (NNSA, 1998). Manganese acts as a cofactor and constituents of several enzymes involved in metabolic processes necessary for the skeletal development, reproductive function and growth. It is a cofactor of oxidative phosphorylation enzymes whose activity increases insulin secretion to improve glucose tolerance under diabetic condition (Lee et al., 2013). This element is also

involved in urea formation, metabolism of amino acids, cholesterol and carbohydrates (Zablocka-Slowinska and Grajeta, 2012).

B. abyssinica provides zinc (Zn) content (5.77 mg/100g) which is within the RDA of 4-14 mg/day in children and adults, respectively (NHMRC, 2005). The presence of Zn suggests the antioxidant and antidiabetic potential of this plant as observed in Chapter 3 and 4. Zn is a critical micronutrient required for structural and functional integrity of biological membranes, maintaining homeostasis, regulation of insulin production, regulation of glucose utilization by muscles and fat cells and detoxification of free radicals (Myers et al., 2012).

The copper (Cu) content (0.93 mg/100g) was also within the RDA of 0.7-1.1 mg/day in children and adults, respectively (NHMRC, 2005). Cu is a constituent of key enzymes like cytochrome c oxidase, amine oxidase, catalase, peroxidase, ascorbic acid oxidase, among others, and plays role in iron absorption. It is an essential micronutrient for bone development, pigmentation, hair growth, reproductive system, haematologic and neurologic systems (Tan et al., 2006). Therefore, the presence of these macro- and micro-elements could explain the observed antidiabetic, antioxidant and anti-inflammatory activities observed with this plant in Chapter 3 and Chapter 4.

Phytochemicals exhibit their antidiabetic effect by several mechanisms, such as inhibition of carbohydrate metabolizing enzymes, manipulation of glucose transporters, β -cell regeneration, acting as antioxidant and enhancing the insulin releasing activity (Tiwari and Rao, 2002). In the present investigation, both acetone and aqueous extracts of *B. abyssinica* effectively inhibited α -amylase and α -glucosidase as described in Chapter 3. The enzyme inhibitory property of these extracts could be ascribed to the phytochemicals identified in this study (Chapter 5; Figure 1). Plant remedies inhibit α -amylase activity due to the presence of flavonoids, flavanols (such as

quercetin and myricetin) and hydrolysable tannins (Ahmed et al., 2011). The strong inhibition of α -glucosidase by *B. abyssinica* extracts could also be due to the phytochemical classes present (Kazeem et al., 2013). Alkaloid, flavonoid, tannic and polyphenolic compounds have been shown to be potent α -glucosidase blockers and several compounds with this hydrocarbon skeleton have been elucidated (Zhang and Yan, 2009; Kazeem et al., 2013). The present study is in agreement with earlier reports that phytochemicals are mild inhibitors of α -amylase and strong inhibitors of α -glucosidase activity (Oboh et al., 2012). The large class of terpenes observed with this species in this Chapter could also suggest the high degree of α -glucosidase inhibitory activity shown with this plant. Terpenes especially the sesquiterpene compounds possess this activity (Yin et al., 2014).

The antioxidant potential of this species as described in Chapter 4 could be hypothesized by the presence of phytochemicals. Phenolic compounds and flavonoids are the major constituents in most plants reported to possess free radical scavenging activity (Williams et al., 2004). The mode of action of phenolic compounds in free radical mopng activity is via inactivating lipid free radicals, or by preventing the decomposition of hydroperoxides into free radicals (Sharma and Singh, 2012). Flavonoids (largest class of phenolics) possess antioxidant properties. The functional hydroxyl groups in flavonoids mediate their antioxidant effects through scavenging free radicals by chelating metal ions, quenching, up-regulating or protecting antioxidant defenses (Sharma and Singh, 2012).

Proanthocyanidins are a class of nutrients that belong to the flavonoid family; hence their mechanism of action as antioxidants is the same as that of flavonoids (Goncalves et al., 2005). The antioxidant action of these phenolic compounds is due to their high tendency to chelate metals. They may inactivate Fe ions by chelating and additionally suppressing the superoxide-

driven Fenton reaction, which is the most important source of reactive oxygen species (Goncalves et al., 2005).

Alkaloids have also been shown to have antioxidant properties via alleviating H₂O₂-induced oxidative damage. For example, four alkaloids namely vindoline I, vindolidine II, vindolicine III and vindolinine IV isolated from *Catharanthus roseus* (L.) G. Don have antioxidant potential with inducing high glucose uptake in the cells; hence also possess antidiabetic effect (Tiong et al., 2013). Flavanols also have free radical scavenging potential. For example, the quercetin 3-O-glucuronide and quercetin 3-O-rutinoside isolated from green beans are potent inhibitors of lipid peroxidation with the antioxidant activity due to the sugar substitutions in the phenolic C ring (Plumb et al., 1999). Saponins have also been proven to have antioxidant potential through hydrogen peroxide scavenging capability (Chen et al., 2014). Tannins are potent primary and secondary antioxidants, whose bioactivities are through chelating metal ions such as Fe (II) and interfering with one of the reaction steps in the Fenton reaction and thereby retard oxidation. Tannins also inhibit lipid peroxidation via the inhibition of cyclooxygenase enzyme (Chen et al., 2014).

The volatile oil analysis revealed presence of compounds with therapeutic activities of interest to this study (Table 5; Figure 2). Some of these compounds could suggest the observed antioxidant activities observed in Chapter 4. For example, α -Terpineol suppresses superoxide production from the cells, attenuating oxidative stress (Brand et al., 2001). α -Terpinolene which used as an anticancer agent has been shown to have strong antioxidant activities (Aydin et al., 2013). Eucalyptol has remarkable antioxidant potential against ferric ions (Zengin and Baysal, 2014). γ -Elemene, γ -Terpinene and 2-Carene also been shown to have antioxidant properties (Foti and Ingold, 2003; Emamia et al., 2011; Akpuaka et al., 2013).

The anti-inflammatory potential of *B. abyssinica* described in Chapter 4 could be due to these plant metabolites capable of inhibiting the inflammation processes (Murugan and Parimelazhagan, 2014). Studies have shown that phenolics and flavonoids act as excellent anti-inflammatory agents. Flavonoids are reported to have anti-inflammatory activities and act through inhibiting a number of immune system inflammatory mediators (Souto et al., 2011). Alkaloids may also inhibit inflammation through blocking the cyclooxygenase and lipoxygenase metabolic pathways of arachidonic acid metabolism (Chao et al., 2009). Proanthocyanidins also possess anti-inflammatory and immunomodulatory properties which could help to prevent oxidative stress related diseases (Majumder et al., 2008).

Some compounds identified in this Chapter (Table 5) have previously been associated with anti-inflammatory activities and could suggest the anti-inflammatory activities observed in Chapter 4. For example, Terpinen-4-ol, a monoterpene alcohol, suppresses pro-inflammatory mediator production by activated human monocytes (Hart et al., 2000). α -Terpineol acts as an anti-inflammatory agent via superoxide production inhibition hence regulating cell functions (Held et al., 2007). γ -Terpinene also has anti-inflammatory activities (Nicholson and Gobert, 2006).

The acetone and aqueous extracts of this plant also showed activity against growth of some pathogenic bacteria (*S. aureus*, *P. vulgaris*, *S. flexneri* and *E. faecalis*) and fungi (*M. canis* and *M. gypseum*) that cause infections in diabetic hosts as described in Chapter 5. The antimicrobial activity could also be ascribed to plant's metabolites observed in this Chapter (Mahboubi et al., 2014). The total phenolics, the highest phytochemicals present in this study (Figure 1) have been reported to have antibacterial activity (Mahboubi et al., 2014). Alkaloids also exert antibacterial activity, for example, an alkaloid-enriched extract from *Prosopis juliflora* pods has activity against *Staphylococcus aureus* (Dos Santos et al., 2013). Flavonoids also possess antifungal and

antibacterial activity as observed with this study (Cushnie and Lamb, 2005). The antibacterial mechanisms of action of selected flavonoids are attributed to inhibition of DNA gyrase, cytoplasmic membrane function and licochalcones A and C energy metabolism (Dos Santos et al., 2013). Proanthocyanidins are also known to be targets as antibacterial agents (Mayer et al., 2008).

Antibacterial activity of saponins from different plant sources have been widely reported (Avato et al., 2006). Tannins act as natural antibiotics through preventing lipid peroxidation or by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells (Avato et al., 2006). Tannins have been further shown to confer inhibitory growth activity against *S. aureus*, *P. aeruginosa* and *E. faecalis* (Min et al., 2008).

The essential oil of *B. abyssinica* also exhibited growth inhibition of some opportunistic bacteria (*S. flexneri*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *P. vulgaris*) and fungi (*T. rubrum*) in diabetic status as described in Chapter 5. The class of terpenes observed in this volatile oil may suggest its antimicrobial activity. Studies have shown that α -Pinene possesses antibacterial and antifungal activities (Rivas da Silva et al., 2012). β -Phellandrene in combination with other monoterpenes have activity against bacteria (Buitrago et al., 2012). Kisko and Roller (2005) have shown that cymene, a monoterpene is an antibacterial agent. Eucalyptol, a monoterpene, possesses antibacterial activity (Zengin and Baysal, 2014). γ -Terpinene has antibacterial and antifungal properties against various human pathogens (Mondello et al., 2006; Oyedemi et al., 2009). γ -Terpinene and α -Terpineol possess bactericidal activity against *Streptococcus pyogenes* and *Proteus vulgaris* (Oyedemi et al., 2009b). α -Terpineol in combination with other compounds has antifungal activity (Pitarokili et al., 2002). Terpinen-4-ol, both in aqueous solutions and vapour form acts as antimicrobial agent against bacteria of *Legionella* genus (Mondello and

Ricci, 2012). Studies have shown that (-)-Globulol can inhibit the mycelia growth hence arrest fungal growth (Tan et al., 2008). Therefore, these classes of phytochemicals and some of the compounds present predict the antimicrobial activity observed with *B. abyssinica*, and marks it as a promising prospect for future synthesis of new antibacterial and antifungal agents (Min et al., 2008).

In conclusion, *B. abyssinica* possesses phytochemicals known to have antidiabetic activities observed in this study. It is composed of large amounts of Terpinen-4-ol, γ -Terpinene, α -Terpinolene and α -Terpineol as the major compounds which have been largely implicated in antioxidant, anti-inflammatory, antifungal and antibacterial activities suggesting their role in *B. abyssinica* therapeutic potentials. Even, though these phytochemicals and their specific compounds are known to have antidiabetic, antioxidant, anti-inflammatory, antifungal and antibacterial properties independently, it is postulated they could be acting singly or in combinations to attenuate the plants' potentials (Ashafa et al., 2010). Therefore, it is crucial to isolate, elucidate the bioactive compounds and determine their pharmacological properties. Characterization of the unknown esteric compounds with their bioactivity warrants more research. This should facilitate the identification of novel antidiabetic, antioxidant, antimicrobial and anti-inflammatory agents. Moreover, the presence of sufficient amounts of proximate, phytonutrients, macro- and micro- elements indicate that this plant is a good source of these nutrients hence; it can be useful in nutritional supplementation.

These findings also provide some scientific evidence for the biological activities of *B. abyssinica* and also accounts for the multi-pharmacological use of this plant in traditional medicine in the management of diabetes mellitus and associated complications such as infections.

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CHAPTER 7

**ULTRASTRUCTURAL MORPHOLOGY OF *BULBINE ABYSSINICA* GROWING IN
THE EASTERN CAPE PROVINCE, SOUTH AFRICA.**

CHAPTER SEVEN

Ultrastructural morphology of *B. abyssinica* A. Rich. growing in the Eastern Cape Province, South Africa.

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Abstract

The genus *Bulbine* (Asphodelaceae) comprises about 40 species in South Africa. *Bulbine abyssinica* is a succulent member of the genus that occurs from the Eastern Cape, through Swaziland, Lesotho, and further north to Ethiopia. The species is often used in traditional medicine to treat rheumatism, dysentery, bilharzia and diabetes. Despite its ethno medicinal value, not much information on the micro-morphological features is available in literature. The present study was undertaken to examine the ultra-morphological features of the leaf, stem and root of the plant using light and scanning electron microscopes. The elemental compositions of the plant parts were studied using energy dispersive x-ray spectroscopy. The mean length and width of the guard cells in the abaxial surface are 0.15 ± 0.002 mm and 0.14 ± 0.002 mm, respectively while those of the adaxial surface are 0.14 ± 0.001 mm and 0.12 ± 0.001 mm, respectively. Electron microscopy revealed the presence of crystals in the leaves, stems and roots. The EDXS microanalysis of the crystals revealed the presence of sodium, silicon, potassium and calcium as the major constituents. The leaf also showed the presence of iron and magnesium, while the stem had aluminium, phosphorous and magnesium. The X-ray analysis of the roots also revealed the presence of sulphur and aluminium. The presence of these elements, which are vital in maintaining good health status, suggests the potential role of *B. abyssinica* in the treatment of infections and some chronic diseases, especially diabetes mellitus.

Key words: *B. abyssinica*; scanning electron microscopy; X-ray spectroscopy; mineral elements.

Introduction

The use of plant materials for the treatment of various diseases is very common in African countries. In traditional African medicine, various *Bulbine* species are used to treat a number of conditions including sexually transmitted diseases, wound infections, dysentery and urinary tract infections (Van Staden and Drewes, 1994; Wanjohi et al., 2005).

The genus *Bulbine* (Asphodelaceae) comprises about 40 species in South Africa. These plants are mostly herbs with leaves that are evergreen and succulent in appearance. They have thick fleshy tuberous roots and are easy to grow (Wanjohi et al., 2005). *Bulbine* species are commonly used by traditional healers in South Africa in the treatment of wounds, burns, rashes, itches, ringworm, cracked lips and herpes (Hutchings et al., 1996).

Bulbine abyssinica is a succulent, perennial herb with a rhizomatous base which grows in small clusters. The plant is a hardy, water-wise plant that offers a brilliant yellow display when in flower. Both flowers and fruit have an attractive bicolored (yellow and black) appearance (Pooley, 1998). The roots are many, slender or swollen. It has soft, dark green leaves which are grass-like and up to 350 mm long. Mature fruits are black, 4mm in diameter and often covered with the faded perianth persisting as a cap. *B. abyssinica* occurs from the Eastern Cape, through KwaZulu-Natal, Swaziland, Lesotho, Free State, North-West, Gauteng, Mpumalanga, Limpopo and further north to Ethiopia (Pooley, 1998).

B. abyssinica is often used in traditional medicine to treat rheumatism, dysentery, bilharzia and cracked lips (Wanjohi et al., 2005). In South Africa, the whole plant is used by traditional healers in the management of diabetes mellitus (Oyedemi et al., 2009).

The stem and root of *Bulbine* species are known to contain anthraquinones such as chrysophanol and knipholone which have anti-bacterial properties (Van Staden and Drewes, 1994; Van Wyk et al., 1995). Some anthraquinones have been isolated from the roots of *B. abyssinica* (Dagne and Yenesew 1994; Bezabih et al., 1997). From the fruits of *B. abyssinica*, three new dimeric anthracene derivatives namely; abyquinone A, abyquinone B and abyquinone C have also been isolated (Wanjohi et al., 2005). Though advances have been made to scientifically validate the use of the plant in traditional medicine, their medicinal value remains obscure.

Production of such compounds with therapeutic properties has been reported in many plant species. In most cases, the source of these bioactive compounds has been attributed to the trichomes (Afolayan and Meyer, 1995). In addition, among the various cell contents, crystals such as calcium oxalate of different types are found in different organs of the plant. Such crystals occur in almost every part of the plant, including both the vegetative and reproductive organs (Prychid and Rudall, 1999).

Crystals such as calcium oxalate play key roles in plant's defense. They may also be waste products as a result of crassulacean acid metabolism, an adaptive feature of plants in arid habitats (Franceschi and Nakata, 2005; Badmus and Afolayan, 2010). Some crystals mediate in reducing the transpiration rate of the leaf and wilting, hence preventing excessive water loss during dry spells (Wintola and Afolayan, 2013). Crystals have been reported to have great therapeutic value including treating cuts, healing wounds and also in fracture management. Presence or absence of crystals, and their dimensions are useful in correct identification of crude drugs and important in detection of adulterants (Masram and Harisha, 2012). Therefore, developmental and structural studies of these trichomes and crystalline

bodies, according to Afolayan and Meyer (1995) and Masram and Harisha (2012) can shed light on the nature of these secreted materials and their functional significance.

Despite the pharmacological and therapeutic uses of *B. abyssinica*, to the best of our knowledge, no information on its ultrastructural morphology is available in literature. Therefore, the objective of this study was to examine the ultrastructural morphology and elemental compositions of the leaves, stems and roots using scanning electron microscope (SEM) and to relate the findings to their possible functional role in the production of therapeutic compounds.

Materials and Methods

Plant material

The leaves, stems and roots of *B. abyssinica* were collected from lower Ncera location in Nkonkobe Municipality of the Eastern Cape Province, South Africa. Lower Ncera is situated between latitude 30° 00 to 34°15'S and longitude 22° 45' to 30° 15'E (Afolayan and Wintola, 2014). It is bounded by the sea in the East and the drier Karoo (semi-desert vegetation) in the West. The elevation ranges from sea-level to approximately 2,200 m in the north and the vegetation is veld type, known as the Eastern Cape thorn veld (Masika and Afolayan, 2003). The voucher specimen (voucher no. KibMed 2014/01) was deposited in the Giffen's herbarium, University of Fort Hare, South Africa for authentication.

Light Microscopy

Microscopic examinations of the epidermal parts of the leaf were carried out according to the procedure of Ogunkunle and Oladele (2008); Otang et al. (2014). Leaf samples of 1 to 3 cm were sectioned from the mid portion of the adaxial and abaxial surfaces of mature leaves

using a razor blade. The sections were washed with distilled water for 2-3 minutes. These sections were placed on clean glass slides with 1-2 drops of distilled water, covered with a cover slip and observed under a Motic light microscope. The microphotographs were taken with a digital camera that was fitted to the light microscope (Akyol, 2014). The stomata density was estimated at X10 magnification. These values were converted to stomata per mm² (Wilkinson, 1979). The density of the epidermal cells, guard cell length and width, guard cell indices were estimated according to the method of Wintola and Afolayan (2013).

Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

Fresh leaves, stems and roots were cut into segments (both transverse and longitudinal sections) of 4-6 mm in length and fixed in 6% glutaraldehyde with pH 7.3 for 12 h. The sections were rinsed with 0.05 M sodium cacodylate buffer (pH 7.5). Each section was then rinsed in distilled water and dehydrated in a graded series of ethanol 10-100% for 20 min per rinse. The sections were dried in a Hitachi HCP-2 critical point dryer and mounted on aluminium stubs with double-sided carbon coated sputter coating with gold palladium (Elko IB-3 Ion Coater). The sections were examined at varying magnifications using JEOL (JSM-6390LV) scanning electron microscope (SEM) set at 10-15 kV accelerated voltage. The energy dispersive X-ray spectroscopy (EDXS) involved both fixing and dehydration procedure as in SEM, while the elemental analysis was done using energy dispersive x-ray analyser which was coupled to SEM, manufactured by Thermo Electron Corporation, 6733B-IUUSN, USA. The Noran system six software was used for imaging.

Results and Discussion

Different features were observed in the leaves of *B. abyssinica*. The mean stomata densities on the abaxial and adaxial surfaces were 21.53 ± 1.68 and 9.81 ± 1.01 per mm², respectively.

This amphistomatic feature shows the paracytic stomata surrounded by the guard cells. The stomata are embedded within the epidermal layer with two subsidiary cells surrounding each stoma (Figure 1A and B). The epidermal cells are symmetric rectangular in shape with wave-like cell walls on both surfaces (Figure 1C and D). The density of the epidermal cells was not statistically different in the lower and upper surfaces, that is, 21.74 ± 0.85 and 21.94 ± 1.36 per mm^2 , respectively.

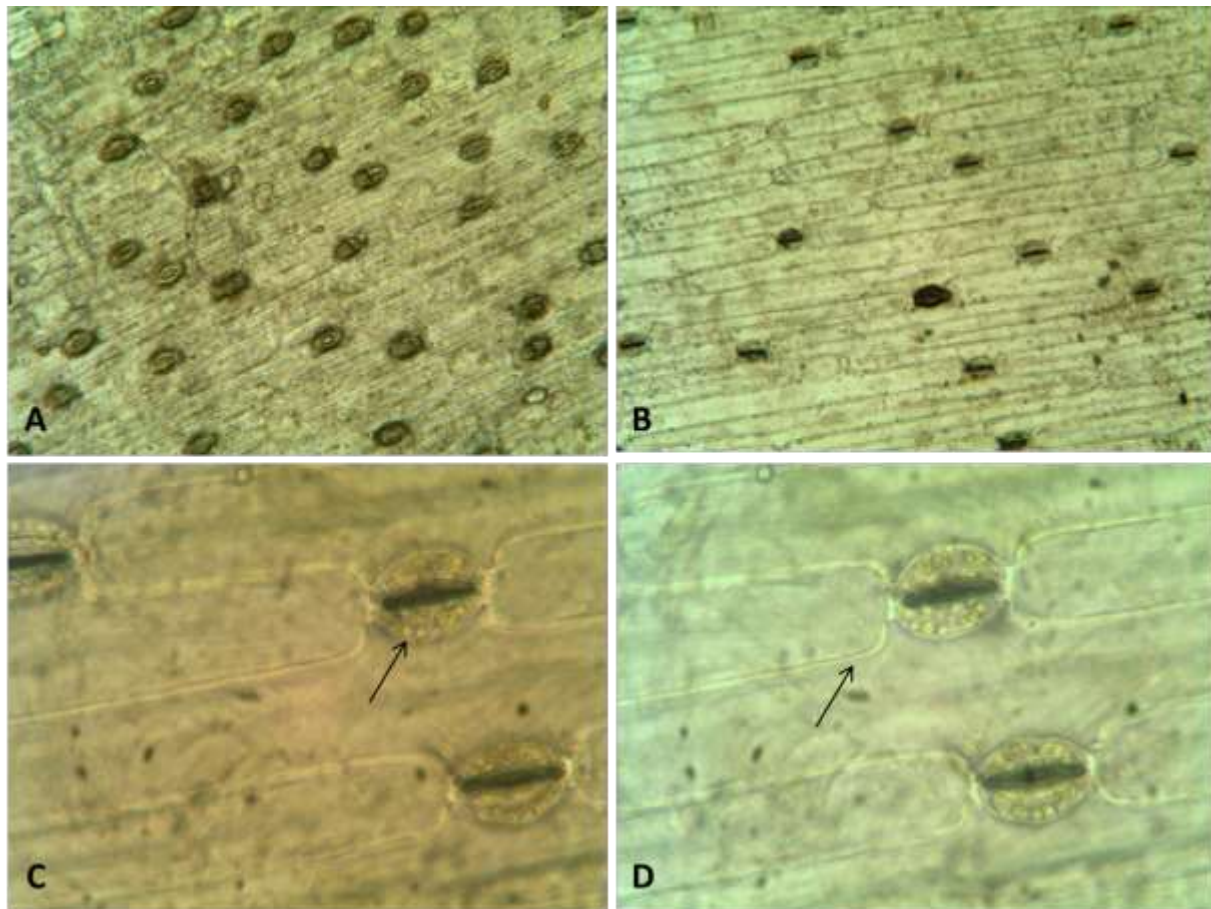


Figure 1: Ultra morphological features of the leaf of *B. abyssinica* (A) stomata distribution on the abaxial leaf surface (10X); (B) epidermal cells and stomata distribution on the adaxial leaf surface (10X); (C) Guard cells and dumbbell stomata (arrow) in the adaxial leaf surface (40X); (D) Rectangular epidermal cells (arrow) on abaxial leaf surface (40X).

The guard cells are dumbbell shaped outlined by a thick inner and thin outer walls which are horizontally embedded to the subsidiary cells (Figure 2A and 2B). The mean length and width of the guard cells on the abaxial surface are 0.15 ± 0.002 mm and 0.14 ± 0.002 mm respectively, while those of the adaxial surface are 0.14 ± 0.001 mm and 0.12 ± 0.001 mm respectively. The guard cell index is $1.67 \times 10^{-8} \mu\text{m}^2$ and $1.30 \times 10^{-8} \mu\text{m}^2$ in the abaxial and adaxial surfaces, respectively. There is no statistical difference in the guard cell indices and mean length and width of the guard cells in both abaxial and adaxial surfaces ($p \leq 0.05$).

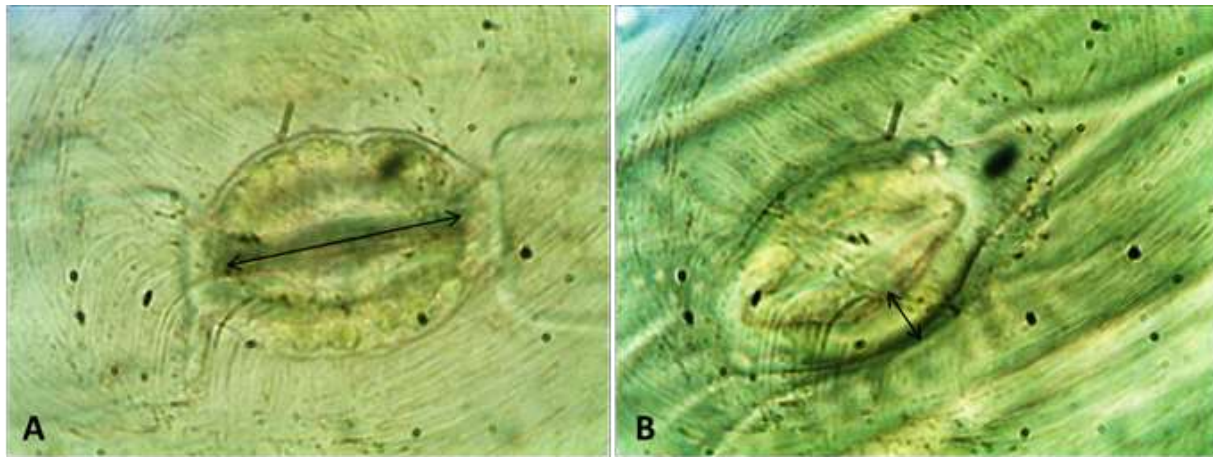


Figure 2: Stoma, dumbbell shaped guard cell, (A) abaxial surface (100X); (B) Adaxial surface (100X).

The micromorphology of the leaf and root section of *B. abyssinica* as seen under SEM is presented in Figure 3A to D, showing the distinctive stomata with the presence of mineral crystals positioned in the stomata pores, scattered in the intercellular spaces within the plant surface and inside the root cavities.

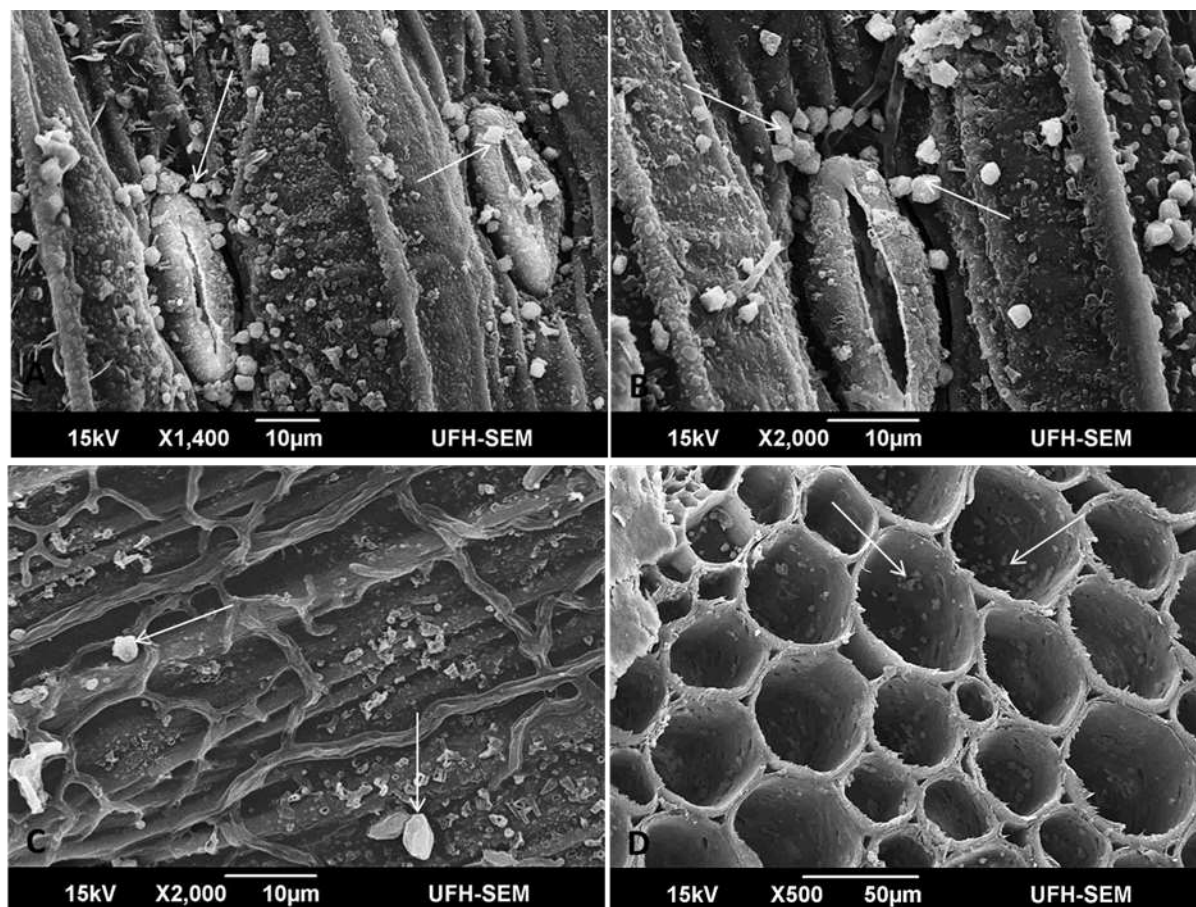


Figure 3: Scanning Electron Micrograph of *B. abyssinica* leaf and root (A) Crystal deposit on the abaxial surface the leaves; (B) concentrated crystals within the stoma on the adaxial surface; (C) fragments of crystals scattered in abaxial surface; (D) crystal deposits in the root cross section.

The micromorphology of the stem is presented in Figure 4A and B showing a cross section of the vascular bundle of the xylem (arranged in ringed shapes) and phloem tissues. The xylem tissue serves as a vessel for transporting water and dissolved minerals; and fibres which offer the structural support to the plant. The phloem transports organic substances through the plant. The structure of the stem shows the presence of droplets of mineral elements in the phloem tissue which are translocated to all parts of the plant (Figure 4B).

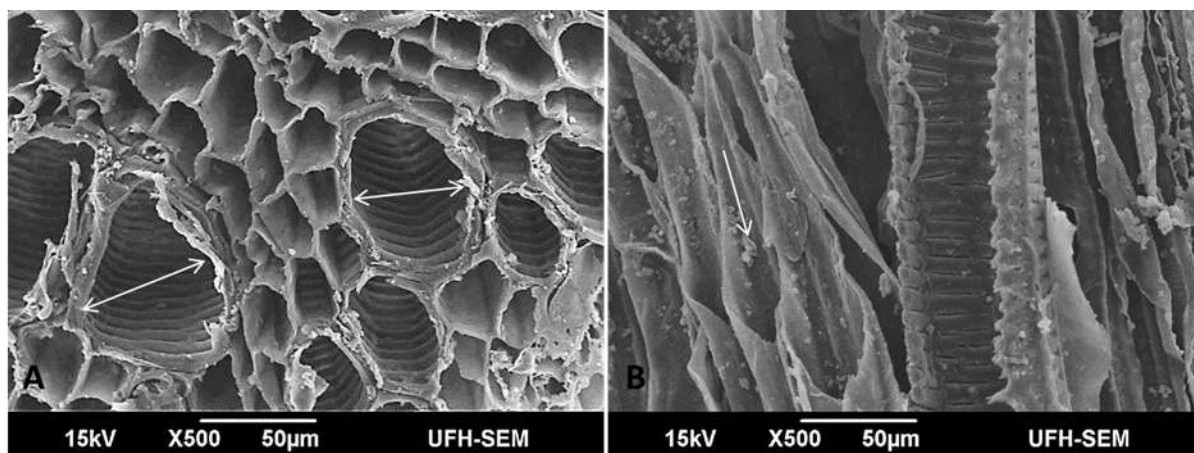
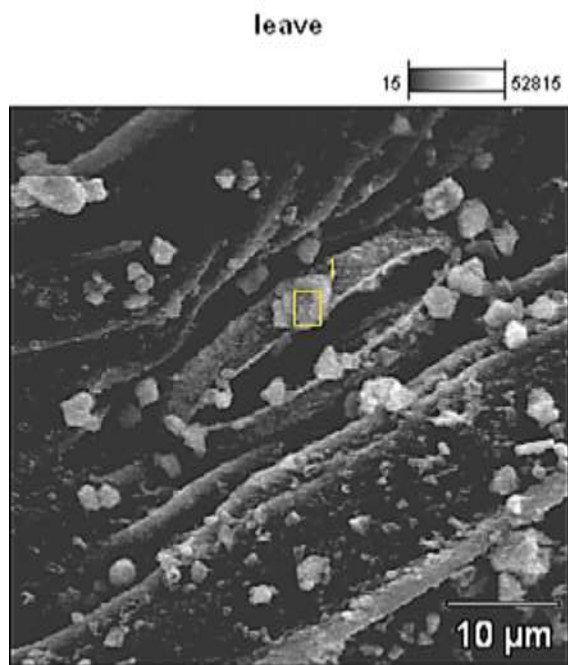


Figure 4: Scanning Electron Micrograph of *B. abyssinica* stem (A) cross sections showing the inner rings of xylem tissues; (B) stem having mineral sediments on the phloem tissue.

The X-ray microanalysis of the leaf, stem and root of *B. abyssinica* generated spectra of the following micro- and macro-mineral elements: carbon (C), oxygen (O), sodium (Na), silicon (Si), potassium (K) and calcium (Ca). The leaf spectra also indicated presence of iron (Fe) and magnesium (Mg). The stem spectra indicated presence of aluminium (Al), phosphorous (P) and magnesium (Mg), while the root had sulphur (S) and aluminium (Figure 5, 6 and 7). Gold (Au) was probably from the spur coater. The mineral constituents of this plant are an indication of its ethno-pharmacological importance. For instance, the high peaks of carbon, oxygen, phosphorus and sodium shows the abundance of these elements while calcium, magnesium, potassium and iron were in moderate quantity. Aluminum, silicon and sulphur were found in small quantity (Table 1).



Full scale counts: 1258

leave_pt1

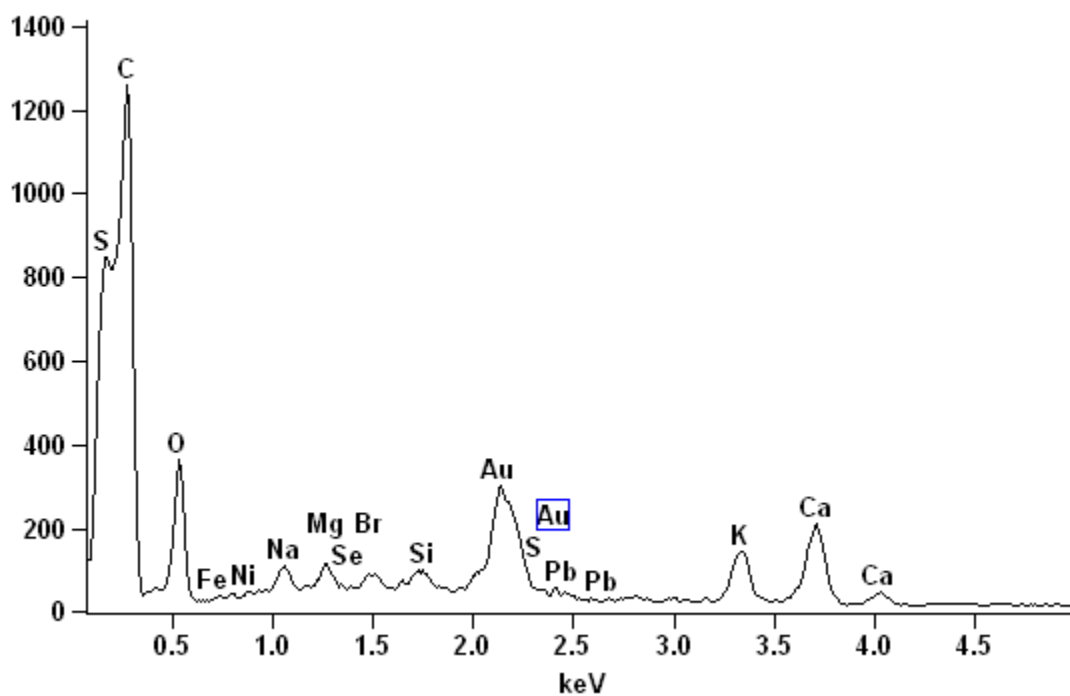
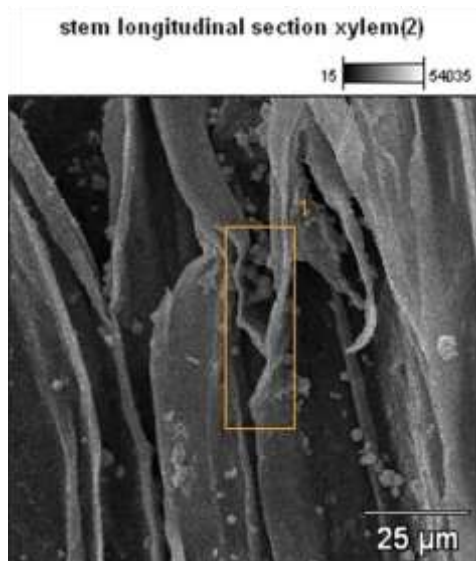


Figure 5: Energy Dispersive X-ray analysis of crystal deposits on the guard cell of *B. abyssinica*; micrograph showing the point of focus of the electron beam.



Full scale counts: 582 stem longitudinal section xylem(2)_pt1

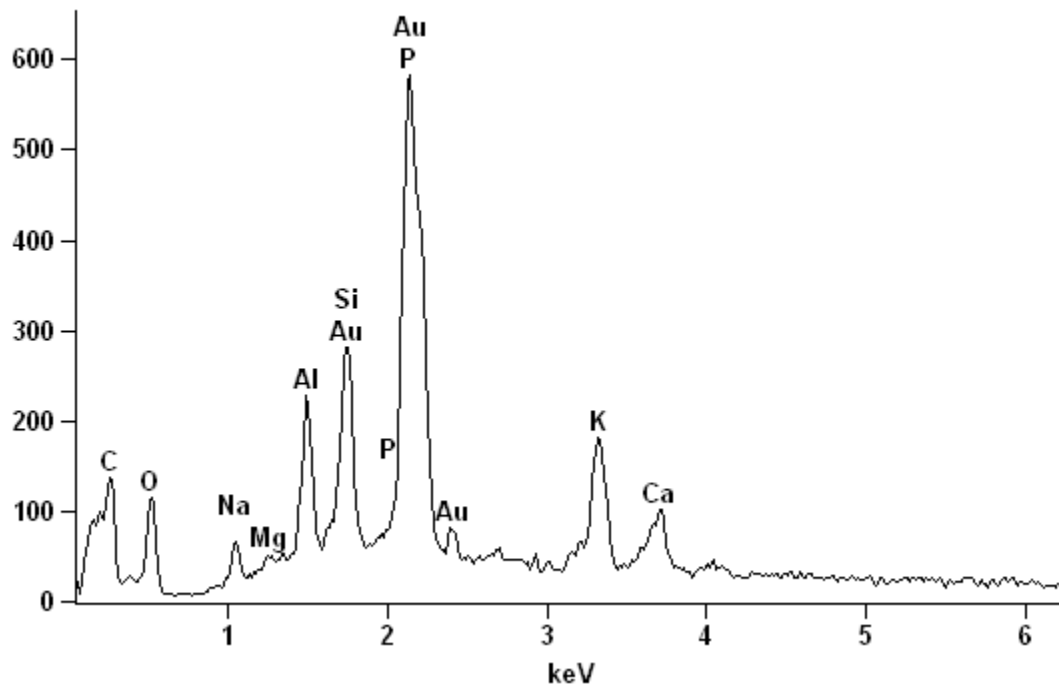
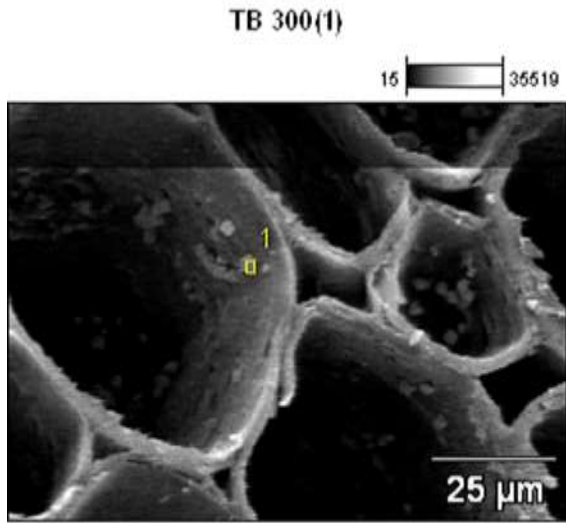


Figure 6: Energy Dispersive X-ray analysis of crystal deposits in the vascular tissue of *B. abyssinica*; micrograph showing the point of focus of the electron beam.



Full scale counts: 1269

TB 300(1)_pt1

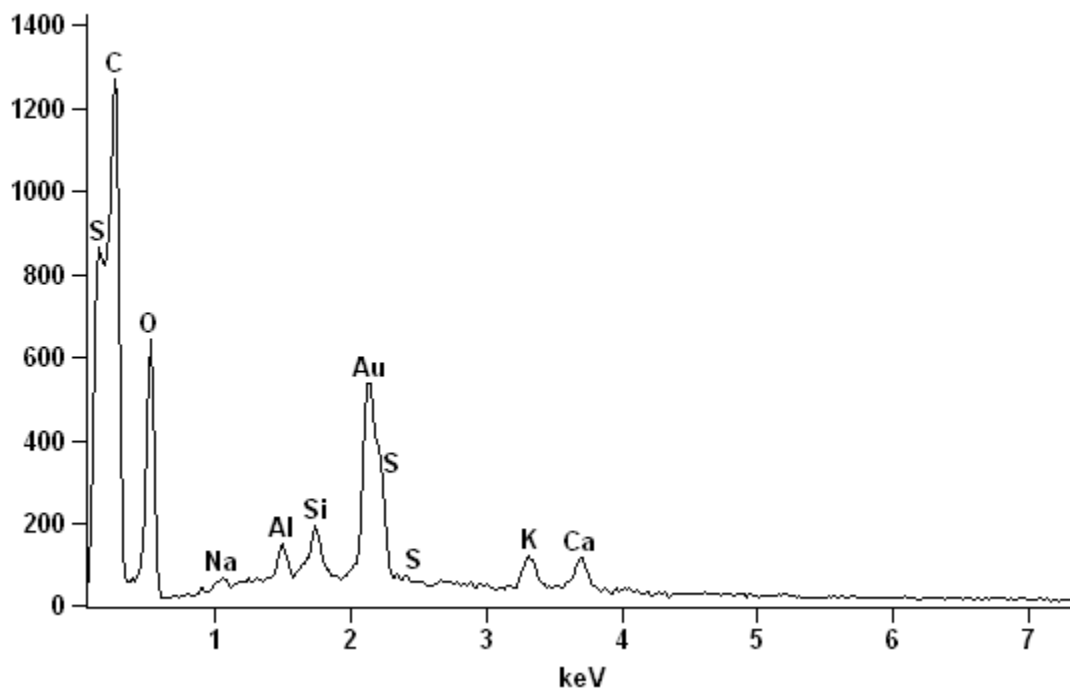


Figure 7: Energy Dispersive X-ray analysis of crystal deposits in the extracellular space of *B. abyssinica* root; micrograph showing the point of focus of the electron beam.

Table 1: Percentage elemental composition of crystal deposits on *B. abyssinica* leaf, root and stem.

Element (%)	Leaf	Root	Stem
Carbon	32.68 ± 0.46	23.92 ± 0.37	2.22 ± 0.15
Oxygen	18.63 ± 0.61	20.34 ± 0.42	4.84 ± 0.29
Sodium	1.22 ± 0.09	0.31 ± 0.05	0.67 ± 0.10
Magnesium	0.92 ± 0.10	0	0.13 ± 0.07
Silicon	0.83 ± 0.07	1.16 ± 0.06	3.26 ± 0.17
Iron	0.03 ± 0.01	0	0
Potassium	1.80 ± 0.10	1.80 ± 0.10	4.79 ± 0.31
Calcium	2.01 ± 0.11	2.01 ± 0.11	2.55 ± 0.16
Phosphorus	0	0	0.09 ± 0.09
Sulphur	0	0.18 ± 0.13	0
Aluminium	0	0.73 ± 0.05	2.17 ± 0.14
Gold	49.56 ± 8.07	49.56 ± 8.07	79.27 ± 7.81

Data are presented as means ± SD of three replicates. The means represented by zero (0) indicate that the mineral element was not detected.

These mineral elements play different metabolic roles in humans. For example, calcium is a key element known in maintaining bones and teeth, regulation of nerve and muscle function. It plays a vital role in enzyme activation during blood clotting (Pravina et al., 2013). Sulfur serves a structural function in cartilage, bone, tendons and blood vessel walls (Parcel, 2002). In addition, sulphur plays a esignificant role in protein synthesis, cell regeneration and blood

cleansing (Afolayan and Otunola, 2014). Potassium is the main intracellular cation in the human body and is required for vital cellular processes. It is involved in regulating acid–base balance, blood pressure, cell membrane function and basic cellular enzymatic reaction (Greenlee et al., 2009; Chatterjee et al., 2011). Magnesium acts as a cofactor to several enzymes like kinase, which participate in energy and protein production processes (Adhikari et al., 2006). It's also vital in strengthening cell membrane structure (Jahnen-Dechent and Ketteler, 2012).

Iron is an important element. It is found in the portion of the cell involved in energy production, neurotransmitter synthesis and in maintaining a stable immune system. Iron functions as haemoglobin in the transport of oxygen (Lieu et al., 2001). Phosphorus is located in every cell of the body and functions as a constituent of bones, teeth, phosphorylated metabolic intermediates and nucleic acids. It serves as buffer in the formation of high energy compounds (Soetan et al., 2010). Sodium is the principal cation in extracellular fluids. It regulates plasma volume and acid-base balance, involved in the maintenance of osmotic pressure of the body fluids, preserves normal function of the nervous and muscle (Constantin and Alexandru, 2011).

Silicon is an essential component of mucopolysaccharides, hyaluronic acid and chondroitin-4-sulfate, which are important constituents of connective tissue. This is a biological cross-linking agent contributing to the structure and resiliency of connective tissue and calcification of bones hence plays a role in wound healing (Price et al., 2013). The presence of these elements accounts for the pharmacological use of *B. abyssinica* in management of diabetes mellitus and complications associated with the disease such as wound healing (Oyedemi et al., 2009).

Conclusion

The present study reveals the micro-morphological characteristics of the leaf of *B. abyssinica* such as amphistomatic epidermal surfaces, rectangular epidermal cells, mean guard cell length and width, subsidiary cells surrounding the stomata. The stoma harbors crystal deposits. The crystal deposits that are also present in the vascular system and extracellular spaces of the roots might suggest that these are the secretory therapeutic products of this plant though their secretory glands still remain unclear. It also shows the presence of crucial micro- and macro-mineral elements which may probably account for its ethnopharmacological importance. This knowledge will help in partially validating the use of the plant in traditional medicine.

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CHAPTER 8

TOXICITY ASSESSMENT OF *BULBINE ABYSSINICA* USING BRINE SHRIMP

CHAPTER EIGHT

Toxicity assessment of *B. abyssinica* using brine shrimp

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Introduction

The use of herbal medicinal products has increased tremendously over the past three decades. Although therapies involving these agents have shown promising potential with the efficacy of a good number of herbal products clearly established, many of them remain untested for their side effects (Okigbo et al., 2009).

In Africa folk traditional medicine, most medicinal plants are indiscriminately used by the healers without knowing their possible side effects (Okigbo et al., 2009). Over recent years, several reports in developing countries indicated adverse effects allegedly arising from the use of medicinal plants (Olajuyigbe and Afolayan, 2012). Some of these effects include abortion of pregnancy, dizziness, vomiting, diarrhea, abdominal pain, fast heartbeat, ulcer, loss of appetite and even death (Elvin-Lewis, 2001). These effects could be attributed to the presence of phytotoxic compounds in the plant extracts (Elvin-Lewis, 2001). In order to minimize this, there is need for a thorough scientific validation on the toxicity of these plants. According to the World Health Organization, the plant remedies used for treatment of various ailments warrant further evaluation for their toxicity potentials (Jamal et al., 2011).

Among these plant remedies is the succulent perennial herb, *B. abyssinica*. The decoction or infusions of the plant parts or the whole plant are widely used in Southern African traditional medicine for the treatment of various ailments including rheumatism, dysentery, bilharzia, cracked lips, infertility, back pain, infections and diabetes mellitus (Wanjohi et al., 2005; Oyedemi et al., 2009).

To the best of our understanding, there was no scientific report on the toxicity potential of the whole plant of *B. abyssinica* to substantiate its traditional usage and guarantee safety to the

users. However, in this study, phytochemical screenings conducted on *B. abyssinica* extracts have demonstrated the presence of total phenols, flavanoids, flavanols, tannins, saponins, alkaloids and proanthracyanidins with strong antidiabetic, antioxidant, anti-inflammatory, antibacterial and antifungal activities (Chapter 3, 4 and 5). The analyses of the volatiles oils have shown presence of terpenes and esters, some of which possess these biological properties (Chapter 6). Some mineral elements and anti-nutrients were detected in this plant (Chapter 6).

Plants containing these phytochemical such as tannins, sapoins and alkaloids have been reported to have toxic side effects in humans (Zeinsteger et al., 2003; Wintola and Afolayan, 2011). Volatile substances like terpenes and indoles parts of the essential oil found in plants have been reported to be cytotoxic (Zeinsteger et al., 2003). Excessive macro- and micro-minerals such as copper, aluminum, zinc, iron and manganese could have deleterious effects (Jose Manuel et al., 2002; Soetan et al., 2010). Phytic acid and oxalate affects metabolism and bioavailability of zinc and calcium, respectively, when present in excess amounts (Akande et al., 2010).

Therefore, the objective of this study was to evaluate the toxicity potentials of the essential oils, acetone and aqueous whole plant extracts of this plant using brine shrimp test (BST) in order to relate the findings to the folklore safe use in the treatment of diabetes mellitus, infections and other diseases.

Materials and Methods

Plant materials

The whole plant parts of *B. abyssinica* including the leaves, flowers, stems and roots were collected and authenticated as described in Chapter 3.

Preparation of extracts

The plant samples were air-dried, ground to homogeneous powder and extracted using acetone and water as described in Chapter 3. For essential oil extraction, the fresh plant samples were properly washed and the essential oil extracted by hydro-distillation, according to the procedure of the European Pharmacopoeia (2002) and as described in Chapter 4. The resulting extracts and essential oils were reconstituted with their respective solvents to give the desired concentrations for further *in vivo* bioassay activities.

Brine shrimp (*Artemia salina*) toxicity test

A. salina hatching and lethality assays were carried out to determine the toxicity of the plant.

Preparation of the assay system

The assay system was prepared as described by Otang et al. (2013). Briefly, 5 petri dishes containing 30 ml of filtered sea water each and a two-fold dilution was set up to yield a series of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg/ml) of the plant fractions. A positive control was prepared in petri dishes by dissolving amphotericin B in seawater (30µl/ml) while petri dishes containing sea water only, served as the blank control. The plant fractions were initially dissolved in 500 µl of their corresponding solvent, and made up to the required volume using filtered seawater. The setup was allowed to stand for 30 min to allow the solvents evaporate (Otang et al., 2013).

***A. salina* hatching assay**

Brine shrimp hatchability assay was evaluated by assessing the hatching success of *A. salina* cysts as described by Otang et al. (2013). Briefly, different concentrations (1 to 0.0625 mg/ml) of the plant fractions and positive control were prepared in filtered sea water. *A. salina* cysts were stocked at a density of 15 individuals per petri dish containing 30 ml of the incubation medium at varying concentrations. The petri dishes were partly covered, incubated at 28°C and under constant illumination for 72 h. The number of free nauplii in each petri dish was counted after every 12 h up to 72 h. The percentage of hatchability was assessed by comparing the number of hatched nauplii with the total number of cysts stocked (Carballo et al., 2003; Otang et al., 2013).

***A. salina* lethality assay**

Brine shrimp lethality assay was evaluated by assessing the mortality *A. salina* nauplii (larvae) as described by Sahgal et al. (2010). Briefly, different concentrations (1 to 0.0625 mg/ml) of the plant fractions and positive control were prepared in filtered sea water. A suspension of nauplii (0.3 ml), containing about 30-35 larvae, was added into each petri dishes, incubated for 28°C and under constant illumination for 72 h. The larvae did not receive food. To ensure that the mortality observed in the bioassay is attributed to bioactive compounds and not to starvation, a blank control (sea water) was added for comparison (Pelka et al., 2000). The petri dishes were then examined and the number of living nauplii (that exhibited movement during several seconds of observation) was counted after every 12h up to 72 h. The percentage of mortality (M %) was calculated as;

$$\text{Mortality (\%)} = (\text{Total nauplii} - \text{Alive nauplii}) \times 100\% / \text{Total nauplii}.$$

All the tests were done in triplicates.

Data analysis

The percentage hatchability success and mortality data obtained from the 5 different concentrations of each fraction and control experiments were used to construct the dose-response curves. These were used to determine their corresponding minimum inhibitory concentration 50 (MIC 50) and lethal dose concentration 50 (LC₅₀) values. The MIC 50 was determined as the concentration of the plant fraction/control drug that inhibited hatching of 50% of the cysts. The LC₅₀ was taken as the concentration required for producing 50% mortality (Syahmi et al., 2010). LC₅₀ values were determined from the best-fit line obtained by linear regression analysis of the percentage lethality versus the concentration. The statistical analysis was done with MINITAB (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used to test the effect of concentration and time of exposure of the plant fractions on the hatchability success and mortality of cysts and larvae, respectively, in comparison to controls (Syahmi et al., 2010). $p < 0.05$ were considered significant.

Results

The hatching success of *A. salina* cysts incubated with different plant fractions and controls are shown in Figure 1. The highest hatching success of 23.6% was observed in cysts incubated with the aqueous extracts of *B. abyssinica*. This was significantly higher than both the positive control (amphotericin B) and the sea water. The essential oil and the acetone extract exhibited more potent inhibitory effects with hatching success of 11.6 and 12.4%, respectively, which was significantly higher than positive control but lower than in sea water.

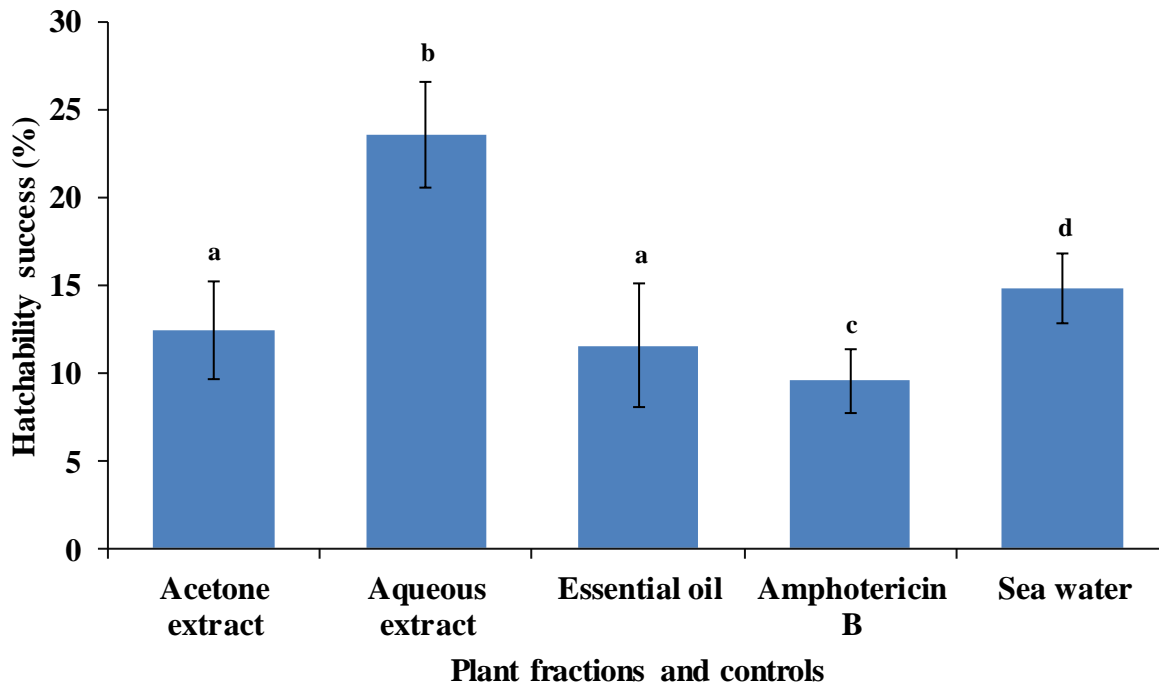


Figure 1: Percentage hatching success of *A. salina* cysts incubated in different plant fractions and controls. The values are means of five concentrations for each plant fraction/control \pm SD of three replicates. Hatching success of fraction/control marked with different letters is significantly different ($p < 0.05$).

The inhibitory effects of the plant fractions and their controls on hatching were estimated and expressed as MIC values. The essential oil and acetone extract exhibited significantly higher hatching inhibitory effects with MIC values of 2.90 and 2.55 mg/ml, respectively, than aqueous extract (3.26 mg/ml) (Table 1).

Table 1: Hatchability of *A. salina* cysts incubated in different concentrations of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*.

Hatchability assay		
Sample	MIC 50 (mg/ml)	R ² (%)
Acetone extract	2.55 ± 0.03 ^a	86.9
Aqueous extract	3.26 ± 0.02 ^b	86.6
Essential oil	2.90 ± 0.05 ^c	94.1
Positive control	2.96 ± 0.05 ^c	99.8

MIC₅₀ is defined as the concentration (mg/ml) of the plant fractions and positive control (amphotericin B) sufficient to obtain 50% of hatching of *A. salina* cysts. Data are presented as means ± SD of three replicates. Means with the different letter superscript in the same column are significantly different ($P < 0.05$). R² (%) denotes the coefficient of determination of the regression equation.

The hatching success of *A. salina* cysts significantly decreased with increasing concentrations of the plant fractions. The hatching success of cysts incubated with essential oil with increasing concentrations from 0.0625 to 1 mg/ml decreased 24 times than with aqueous extract and positive control which decreased by 4 and 6 times, respectively. The acetone extract elicited 100% hatching inhibition at 1 mg/ml (Figure 2).

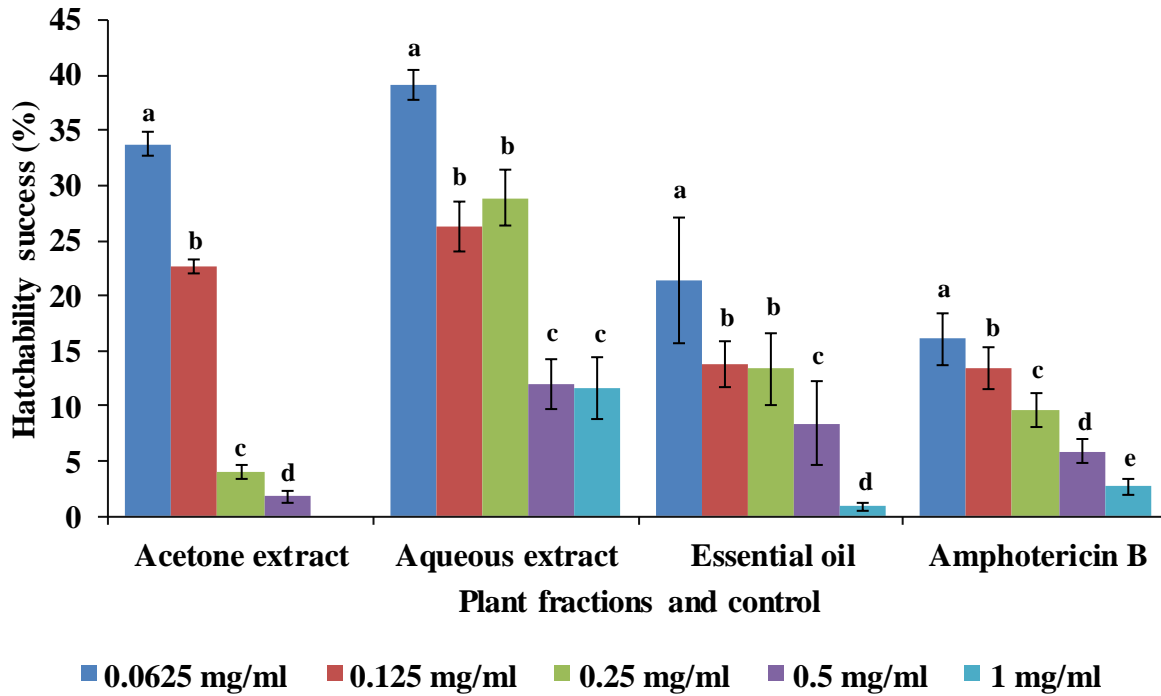


Figure 2: Percentage hatching success of *A. salina* cysts incubated in different concentrations of the plant extracts and control. The values are means for concentrations for each plant fraction/control \pm SD of three replicates. Hatching success of fraction/control in different concentrations marked with different letters are significantly different ($p < 0.05$).

The results from this study showed that after 36 h of exposure, hatching success of the cysts incubated in aqueous and acetone extracts only significantly increased by 1, 1.1 fold, respectively while with essential oil, decreased significantly by 0.7 folds. The increment was significantly lower than when incubated in filtered sea water (5.8 fold higher) within the same duration of time (Figure 3).

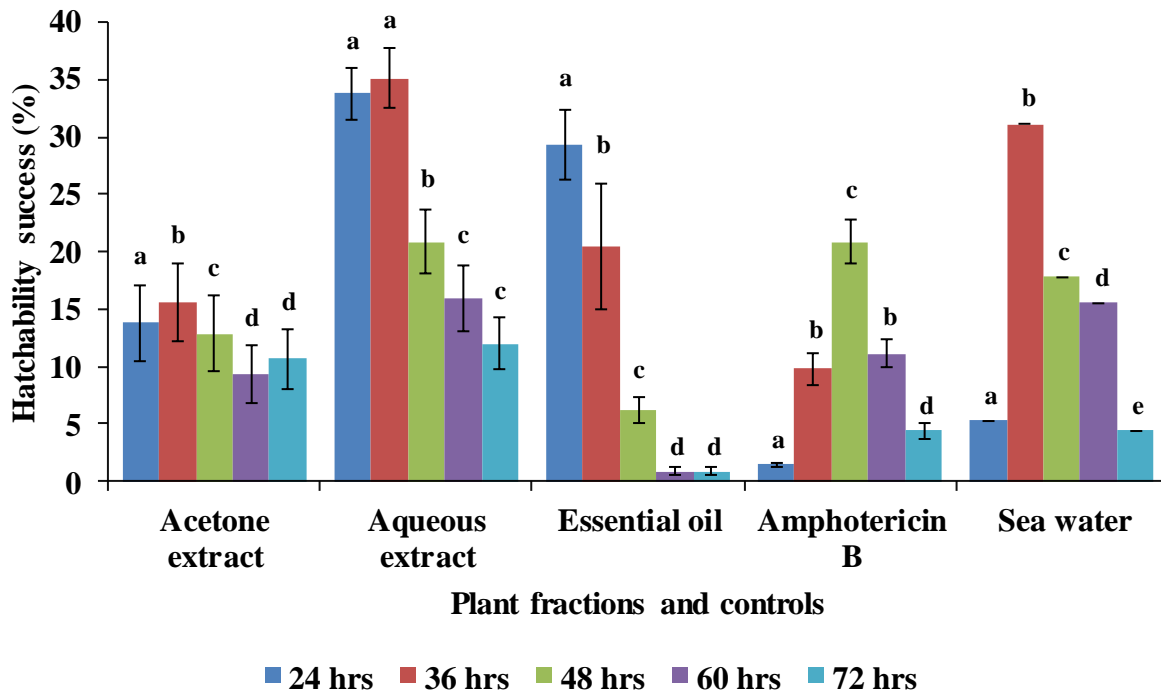


Figure 3: Percentage hatching success of *A. salina* cysts incubated in different durations in the plant fractions and controls. The values are means of experiments for each plant fraction/control \pm SD of three replicates. Duration of time marked with different letters is significantly different ($p < 0.05$).

The effect of varying concentrations of the plant fractions on the mortality of larvae is shown in Figure 4. The degree of mortality of nauplii was in a concentration dependent fashion. The results showed that the mortality significantly increased by 2 folds in all test fractions with increase in concentrations (0.0625 to 1 mg/ml) which was significantly similar to the positive control.

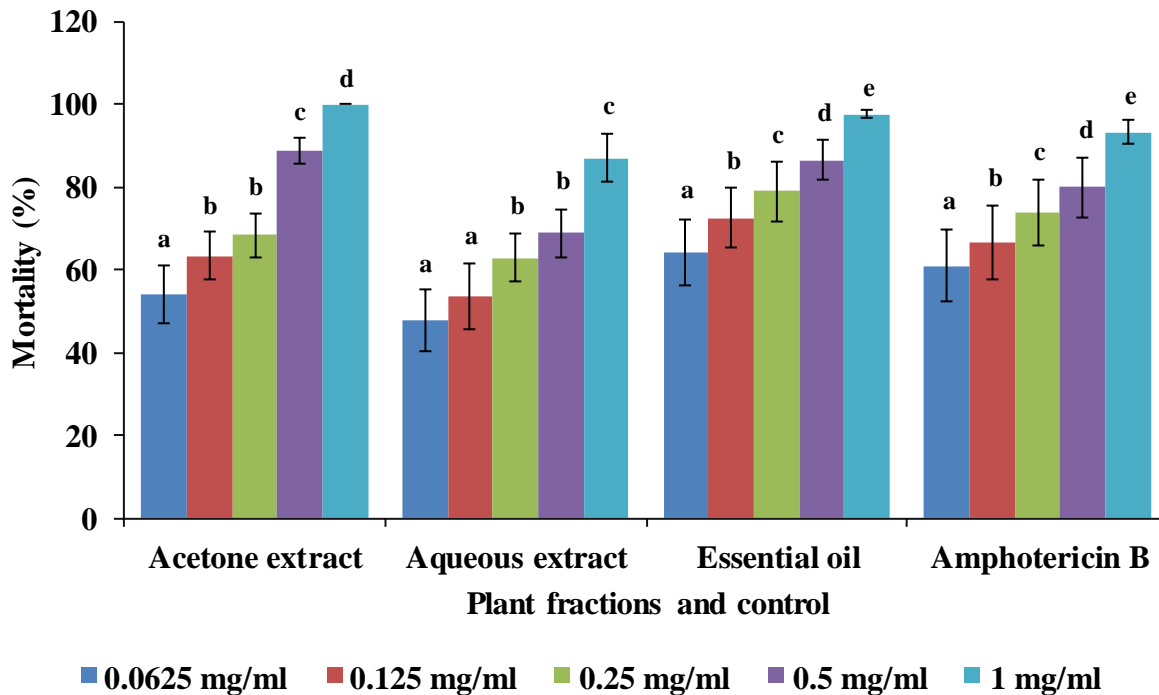


Figure 4: Percentage mortality of *A. salina* nauplii incubated in different concentrations of the plant extracts and control. Mortality (%) is the means of experiments for each plant fraction/control \pm SD of three replicates. Mortality (%) of nauplii in each fraction/control in different concentrations marked with different letters is significantly different ($p < 0.05$).

The results also showed that the mortality of nauplii incubated in these plant fractions increased exponentially with time. After 36, 48, 60 and 72 h of incubation in acetone extract, the mortality significantly increased by 1.1, 1.3, 1.7 and 1.9 folds, respectively. With aqueous extract, the mortality significantly increased by 2.9, 3.1, 4.6 and 5.2 times more after 36, 48, 60 and 72 h, respectively. The nauplii death rate also increased by 2.5, 2.7, 3.1 and 3.1 folds after incubation in essential oil over the same period of time, respectively. The increase in mortality with these plant fractions was significantly higher than the positive control at all duration times (Figure 5).

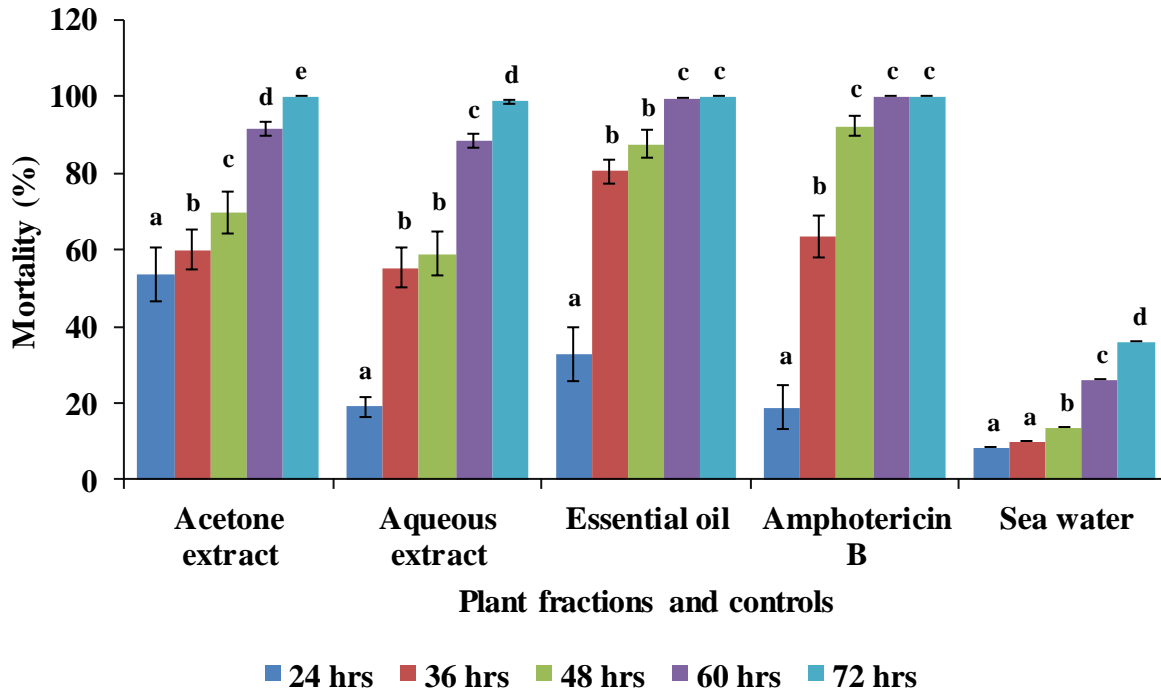


Figure 5: Percentage mortality of *A. salina* nauplii incubated in different durations in the plant fractions and controls. The values are means of experiments for each plant fraction/control \pm SD of three replicates. Duration of time marked with different letters is significantly different ($p < 0.05$).

In overall, the mortality of nauplii was significantly similar when incubated with the essential oil, acetone extract and the amphotericin B which was significantly higher than in sea water. The aqueous extract led to significantly lower larvae mortality when compared to amphotericin B but higher than when larvae are incubated in sea water (Figure 6). The estimated LD₅₀ results were 3.12, 3.18 and 3.36 mg/ml for the essential oil, acetone and aqueous extracts, respectively (Table 2).

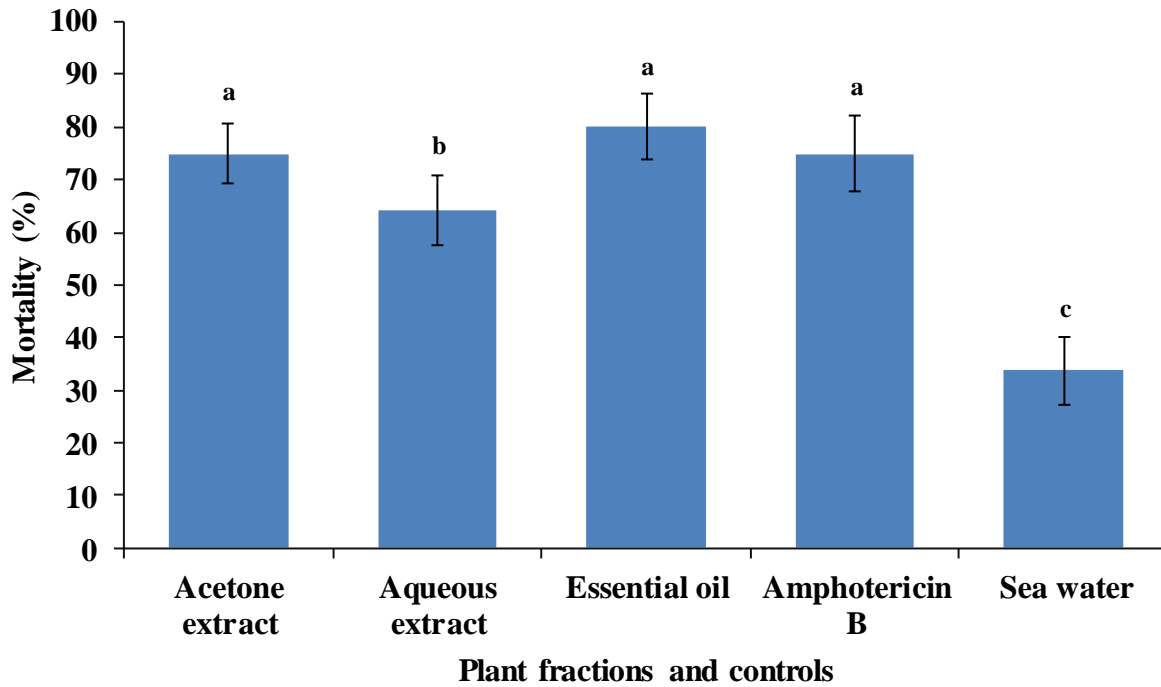


Figure 6: Percentage mortality of *A. salina* nauplii incubated in different plant extracts and controls. The values are means of five concentrations for each plant fraction/control \pm SD of three replicates. Percentage mortality of fraction/control marked with different letters is significantly different ($p < 0.05$).

Table 2: Lethality of *A. salina* nauplii incubated in different concentrations of essential oil, acetone and aqueous whole plant extracts of *B. abyssinica*.

Lethality assay		
Sample	LD ₅₀ (mg/ml)	R ² (%)
Acetone extract	3.18 ± 0.05 ^a	96.0
Aqueous extract	3.36 ± 0.06 ^b	94.9
Essential oil	3.12 ± 0.02 ^c	99.1
Positive control	3.29 ± 0.04 ^d	96.8

LD₅₀ is defined as the concentration (mg/ml) of the plant fractions and positive control (amphotericin B) sufficient to obtain 50% of mortality of *A. salina* nauplii. Data are presented as means ± SD of three replicates. Means with the different letter superscript in the same column are significantly different ($P < 0.05$). R² (%) denotes the coefficient of determination of the regression equation.

Discussion

Medicinal plants are widely used in folk medicine for the treatment of various ailments. As the use of these species increases, there is need to screen for toxicity of the plants to guarantee safety to the users (Nazri et al., 2012). There are various *in vivo* and *in vitro* toxicity assay methods used to validate the safety of plant remedies (Ouedraogo et al., 2012).

The invertebrate, *A. salina* Leach (brine shrimp) test is used as the first line of preliminary evaluation of the general toxicity of herbal remedies. Then the appropriate candidate(s) is/are selected for further tests such as *in vitro* cytotoxicity assays with mammalian cell lines to validate the toxicity results of the brine shrimp test (BST) (Rajabi et al., 2015). BST is widely used for chemicals and natural compounds toxicity studies since it is a rapid,

convenient, low cost and little amounts of test materials are required. *A. salina* cysts are easily available commercially and inexpensive (Otang et al., 2013).

In this study, the toxicity of *B. abyssinica* was evaluated using BST. The toxicity assessment was based on both the inhibition of hatching of the cysts and mortality of the hatched nauplii in different concentrations of plant fractions and controls (Carballo et al., 2003). The hatching success of *A. salina* cysts incubated with different plant fractions showed the highest hatching success of cysts at 23.6% when incubated with the aqueous extracts of *B. abyssinica* (Figure 1). The estimated MIC values to indicate the inhibitory effects of the plant fractions showed that aqueous extract had the highest value of 3.26 mg/ml indicating that this extract could be less toxic and safer among the three fractions.

The hatching success of *A. salina* cysts significantly decreased with increasing concentrations of the plant fractions in a dose dependent manner with the acetone extract eliciting 100% hatching inhibition at 1 mg/ml (Figure 2). This could be explained by presence of toxic metabolites in the fraction or high concentration of the metabolites. The hatching of cysts when incubated at high test concentration (1 mg/ml) with essential oil and aqueous extracts can be predicted to be as a result of tolerance property by cysts at higher concentrations. *A. salina* cysts possess a resistant cyst stage which makes it tolerant to a wide range of pH ranging from freshwater to saturated saline (Caldwell et al., 2003).

In BST, the optimal hatching of cysts to yield a large number of larvae (nauplii) is achieved within 48 h (Meyer et al., 1982). The results from this study showed poor cysts hatching into larvae after 36 h when incubated with the plant fractions (Figure 3). The low hatchability success observed with this plant species could be predicted to the presence of chemical

metabolites which probably altered the development of *A. salina* embryos. Toxins are known to inhibit the progression of early developmental stages of *A. salina* (Vasconcelos et al., 2010).

The *A. salina* resistant cyst stage property at higher salinities makes the hatchability test less desirable assay for preliminary herbal remedies' toxicity test. The use of hatched larvae (nauplii) has been used to by-pass this toxin tolerant cyst stage hence lethality assay involving nauplii was employed (Caldwell et al., 2003; Otang et al., 2013). The exposure of nauplii on different concentrations of the plant fractions was done to extrapolate the baseline of the dosage of the fractions in order to prevent acute overdose in subsequent *in vivo* trials for the plant extracts (Otang et al., 2013). The effect of different concentrations all the plant fractions on the mortality of larvae was in a concentration dependent fashion (Figure 4). Though these are toxicological data, it can be postulated that these plant fractions have pharmacological activity based on the dosage administered (Otang et al., 2013).

The effect of the plant fractions on the nauplii over duration of time was done to establish the maximum sensitivity of nauplii on the toxic secondary metabolites/ chemical compounds present in this plant species. The mortality of nauplii incubated in these plant fractions increased exponentially with time with the highest mortality observed at 72 h with all the fractions. The increment in mortality with these plant fractions was significantly higher than positive control at all duration times (Figure 5). The nauplii attain the second and third instars of their life cycle within in 48 h hence reveal their greatest sensitivity to toxins at this time (Sreejamole and Radhakrishnan, 2013). However, the findings of this study indicate that the maximum sensitivity was reached after 72 h of exposure. This is probably due to presence of nutritive metabolites than toxic chemicals. Moreover, *A. salina* nauplii are known to stay

alive for 48 h without food due to enrichments from the yolk-sac (Pelka et al., 2000). Therefore, the nauplii survival observed after 60 h in this study with all the plant fractions could also be explained by the presence of the non-toxic metabolites in the plant species.

In brine shrimp lethality test, the criterion of toxicity for plant remedies is defined as follows; the plant extract showing LC_{50} values greater than 1000 $\mu\text{g/ml}$ are considered non-toxic, LD_{50} values equal/greater than 500 $\mu\text{g/ml}$ but not greater than 1000 $\mu\text{g/ml}$ are considered to have weak toxicity while those having LD_{50} values less than 500 $\mu\text{g/ml}$ are considered toxic (Meyer et al., 1982; Bastos et al., 2009). In this study, the mortality of nauplii was significantly higher when incubated with essential oil and acetone extract than with aqueous extract of this species with estimated LD_{50} values of 3.12, 3.18 and 3.36 mg/ml, respectively (Figure 6). These estimated LD_{50} values show that these plant fractions exhibited low mortality potential with LD_{50} values greater than 1000 $\mu\text{g/ml}$. Therefore, based on criterion of toxicity, these plant fractions can be regarded as non-toxic (Meyer et al., 1982; Bastos et al., 2009).

The findings from this study have shown that *B. abyssinica* exhibited non-toxic (LD_{50} values greater than 1 mg/ml) threshold with brine shrimp toxicity assays hence can be considered possibly as a safe herbal remedy. However, further *in vivo* and *in vitro* toxicity test are required to validate the safe use of this herbal remedy.

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CHAPTER 9

GENERAL DISCUSSION, CONCLUSIONS AND CONTRIBUTION TO KNOWLEDGE

CHAPTER NINE

GENERAL DISCUSSION, CONCLUSIONS AND CONTRIBUTION TO KNOWLEDGE

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GENERAL DISCUSSION

Diabetes mellitus is an important metabolic syndrome that currently affects more than 180 million people worldwide (Gulati et al., 2012). In Africa, diabetes mellitus is no longer a rare disease. Recent investigations on non-communicable diseases indicated an increase in its prevalence from 1% to 20% of the population in Africa. In South Africa, the current prevalence is between 4% and 6% (Wild et al., 2004). The global prevalence of diabetes has risen drastically over the past two decades and it is postulated to double by 2013 if no action is taken (Gulati et al., 2012). Therefore, this has led to constant re-evaluation of the strategies used in the management of this disease. This is also compelled by the costs and side effects of the synthetic anti-diabetic drugs currently being used. Other drawbacks include insulin resistance, gastrointestinal upsets and lactic acid intoxications (Rabyah et al., 2013).

Herbal therapy is one of the widely used alternative medicines for the management of diabetes. In developing countries about 80% of the populations are now using traditional medicine as their primary health care (WHO, 2002). In South Africa, in particular the Eastern Cape Province, it has a long history of using herbal therapy in the treatment of various diseases including diabetes mellitus (Afolayan and Sunmonu, 2010). The uses of medicinal plants have long played a significant role in the lives of the Xhosa people, the primary inhabitants of the study area. Most rural communities of the region are regularly using wild harvested herbal medicines as an initial response to illness (Dold and Cocks, 2000). Although, there is an increasing interest in the use of medicinal plants for the treatment of this disease, there is need to provide scientific evidence to justify the ethnomedicinal use and the safety to the users. The idea of evaluation of medicinal plants used in folk medicine has been further reinforced by WHO in trying to discover new antidiabetic agents (WHO, 2002).

B. abyssinica is among the plants used by the traditional healers in the management of diabetes (Oyedemi et al., 2009). Thus, the choice of *B. abyssinica* for this study was based on the ethnobotanical information obtained from indigenous Xhosa people of Eastern Cape Province, who have been using a decoction prepared from the whole plant in the management of this disease (Oyedemi et al., 2009).

Antidiabetic activities of the plant

One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycemia. This is achieved by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes including α -amylase and α -glucosidase, in the digestive tract (Deepa et al., 2013). Inhibitors of these enzymes, known as ‘starch blockers’, delay carbohydrate digestion, prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently dampening the post-prandial plasma glucose rise (Kotowaroo et al., 2006). It is hypothesized that these starch blockers attach themselves to the carbohydrate molecule where the α -amylase would normally split the compound to produce dextrans (Kotowaroo et al., 2006). As a result, the carbohydrate is not broken down into glucose and passes out of the body undigested (Kotowaroo et al., 2006). Examples of such inhibitors in clinical use are acarbose, miglitol and voglibose. These possess various drawbacks including gastrointestinal ailments (bloating, abdominal discomfort, diarrhea and flatulence). Therefore, screening of α -amylase and α -glucosidase inhibitors in medicinal plants has received much attention (Telagari and Hullatti, 2015). The α -amylase and α -glucosidase inhibitory potentials of *B. abyssinica* were studied to validate the antidiabetic property of this plant. The findings revealed that the species has mild and

strong inhibition to α -amylase and α -glucosidase activities, respectively, explaining the possible mechanism of action of this plant.

Antioxidant and anti-inflammatory activities of the plant

The role of oxidative stress in the pathogenesis of diabetes mellitus is well established due to increased production of free radicals such as nitric oxide and occurrence of weak antioxidant mechanisms (Matough et al., 2012). Prolonged oxidative stress leads to pancreatic β -cells damage leading to pancreatic dysfunction (Matough et al., 2012). Damaged cells and tissues initiate inflammatory processes and hence predisposal to many diseases including diabetes, atherosclerosis, infections and many others (Pandey and Rizvi, 2009). Plant-based antioxidant and anti-inflammatory agents have taken researchers greater interest since they can relieve oxidative damages caused by free radicals (Pandey and Rizvi, 2009). Exploration into the *in vitro* antioxidant and anti-inflammatory properties of *B. abyssinica* was conducted to understand its mechanism of actions. The analysis revealed that the species has the potential to mop up free radicals including lipid peroxide, nitric oxide and hydroxyl radicals which are deleterious to the cell membranes. If the free radicals escape the antioxidant mopping system, this plant has the potential to prevent cell membrane protein denaturation and/or membrane lysis due to the presence of membrane stabilizers and/or protein denaturation inhibitory agents. Thus anti-diabetic properties of this plant could be partially explained by the strong antioxidant and anti-inflammatory capacity demonstrated in this study.

Antibacterial and antifungal properties of the plant

Antioxidant system failure and inflammatory status in tissues are crucial in the pathogenesis of bacterial and fungal infections as secondary complications in diabetic patients (Hodgson et al., 2014). Some of these infections lead to pulmonary, respiratory and gastrointestinal tract

failure leading to death (Casqueiro et al., 2012). In the treatment of diabetes mellitus, the control of these secondary infections has become apparent as the maintenance of normal glycaemia is needed to sustain normal health (Schaberg and Norwood, 2002). Therefore, there is continued use of antibiotics in the treatment of these infections (Amuka et al., 2013). However, resistance to these antibiotics by the opportunistic bacteria or fungi has increased, leading to use of plant based medicines (Amuka et al., 2013). Consequently, assessment of the antibacterial and antifungal activities of this plant on some selected opportunistic bacteria and fungi isolates was carried out. The findings suggests that this plant has the capability of treating infections caused by bacteria (*Shigelle flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumonia*, *Proteus vulgaris* and *Streptococcus pyogen*) and fungi species (*Microsporium canis*, *Microsporium gypseum* and *Trichophyton rubrum*). Thus, this species can attenuate secondary infections complementing the antidiabetic activity of this plant in the management of diabetes mellitus.

Holistic nature of the plant and identification of bioactive metabolites in the plant

Medicinal plants are sources of pharmaceutical agents (Petrovska, 2012). In the approach to identify the bioactive metabolites of *B. abyssinica* responsible for the antidiabetic, antioxidant, anti-inflammatory, antibacterial and antifungal properties of this plant, a holistic nature of the herbal remedy was analyzed. The findings suggest that this plant is composed of vital proximate, vitamins and macro- and micro-elements whose quantities are sufficient for normal health maintenance. The phytochemical analysis of the plant extract revealed the presence of bioactive metabolites such as tannins, flavonoids, flavanols, phenolics, saponins, alkaloids and proanthocyanidins. These metabolites are known to be biologically active attenuating diabetic condition through different mechanisms (Cummings et al., 2004). These

mechanisms include inhibition of carbohydrate metabolizing enzymes in the gut, enzymatic degradation and scavenging of free radicals that are generated during or at the onset of diabetes, and inhibiting inflammatory processes (Edwin et al., 2008). Further analysis of the plant's volatile oil showed a large class of terpenes and traces amounts of esters whose specific compounds were linked to possessing free radical scavenging activities, anti-inflammatory and antimicrobial properties through relevant literature. Thus, in this study, these metabolites and compounds accounts for the pharmacological activities observed with *B. abyssinica* in relation to its use in diabetes mellitus management.

Ultrastructural morphology evaluation of the plant

Plant bioactive products such as alkaloids, flavonoids and terpenoids have been shown to be produced as secretions by the plant organs. These compounds have therapeutic potentials such as anti-tumor, antimicrobial and antidiabetic activity (Koduru et al., 2006). Glandular trichomes commonly found on the leaf appendages and other plant organs are the major secretory sites of these products (Afolayan and Otunola, 2014). It has also been hypothesized that crystals are other secretory bioactive products with therapeutic value including healing of infections (Masram and Harisha, 2012). The secretory organs' information facilitates efficient extraction and identification of these compounds (Masram and Harisha, 2012). Exploration was done to assess the ultrastructure information of the leaf, stem and roots of *B. abyssinica* to hypothesize the secretory sites of the bioactive therapeutic metabolites observed with this species in this study. The findings indicate presence of crystals in some plant's parts which are composed of essential macro- and micro-elements associated with pharmacological properties of interest in the management of diabetes mellitus. It is

postulated that these crystals are the likely secretory products of the plant though their secretory organs remains obscure.

Brine shrimp toxicity assessment of the plant

The general notion that phytomedicines are completely safe and free from side effects because they come from natural sources is incorrect (Calixto, 2000). There are some risks that come with the use of some medicinal plants due to the metabolites present hence it is essential to understand the toxicity threshold potential of the herbal therapy (Calixto, 2000). Thus, the extracts of *B. abyssinica* used in this study were screened for toxicity using the Brine shrimp toxicity assay. The relative low toxicity exhibited by the volatile oil, acetone and aqueous extract of this plant on the hatching success of *A. salina* cysts and low toxicity indices with the mortality of its larvae raises prospects that this herbal remedy could be potentially safe to the users. However, this warrants further investigations.

CONCLUSIONS

This study has justified the ethno-medicinal usage of *B. abyssinica* for the management of diabetes mellitus. The antidiabetic activity of the plant may be attributed to the presence of phytochemicals which possess carbohydrate metabolizing enzyme inhibitory activities. The phytochemicals and compounds present also contribute to the free radical scavenging activity and anti-inflammatory potential meaning lessening and/or preventing the pathogenesis of diabetes mellitus and its associated complications. The beneficial effect of this plant on the treatment of secondary infections associated with diabetes further gives confidence to the therapeutic usage of this plant as an antidiabetic plant. The proof of the presence of these secretory products in the plant parts through ultra-structural studies provides evidence that this species is a source of these bioactive agents. More so, the presence of crucial nutritive factors, macro- and micro-minerals in

required amounts also support the antidiabetic potential of *B. abyssinica* and can serve as dietary supplements for proper health maintenance. The toxicity evaluation of the plant recommends it to be a safe remedy. These findings provide scientific evidence that justifies the use of this plant in management of diabetes mellitus and other diseases in South Africa folk medicine.

CONTRIBUTION TO KNOWLEDGE

1. For the first time, through a series of laboratory experiments, this study has provided a scientific insight into the possible mechanism of action of *B. abyssinica* as antidiabetic agent and justifies its folklore use in South Africa.
2. The study has identified the metabolites and compounds that could be responsible for the multi-pharmacological potentials of this species which offers a new path in bioprospecting. This may lead to discovery of novel compound(s) that could facilitate development of new affordable drugs.
3. The study has further outlined the nutritive importance and safety of this herbal medicine suggesting a new source of dietary supplement ideal for good health maintenance.

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