# *In vitro* anti-oxidative and carbohydrates digesting enzymes inhibitory effects of some medicinal plants used for the management of diabetes in the Mrewa district, Zimbabwe

**By**

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**Supervisor: Dr M. S. Islam**

## PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School of Life Sciences, Faculty of Agriculture, Engineering and Sciences at the University of KwaZulu-Natal (Westville Campus), Durban, South Africa from February 2012 to November 2013, under the supervision of Dr. Md. Shahidul Islam.

These studies represent original work of the author and have not been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Signature of student…….

Signature of supervisor……...

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DECLARATION 1-PLAGIARISM

I,……...…….……declare that

The research reported in this dissertation except where otherwise indicated, is my original research.

This dissertation has not been submitted for any degree or examination at any other university.

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**DECLARATION 2: PUBLICATIONS & PRESENTATIONS**

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, in press and published and give details of the contributions of each authors to the experimental work and writing of each publication).

In all the publications included in this thesis, I designed the work, carried out the experiments and also prepare the first drafts of all the manuscripts. The co-authors assisted in troubleshooting during experiments, analysis of data and in with proofreading and editing of manuscripts.

**PUBLICATIONS**

**Publication 1**:

**Talent Chipiti, Mohammed Auwal Ibrahim, Neil Anthony Koorbanally and Md. Shahidul Islam**.:"I*n vitro* antioxidant activities of leaf and root extracts of *Albizia antunesiana* harms". **Acta Poloniae Pharmaceutica**, 70(6): 1035-1043 (2013).

**Publication 2**:

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**Manuscript 1**:

**Talent Chipiti, Moganavelli Singh, Mohammed Auwal Ibrahim and Md. Shahidul Islam** "*In vitro* α-amylase and α-glucosidase inhibitory effects and cytotoxicity activity of ethanol and aqueous extracts of *Albizia antunesiana* harms parts", **(** In preparation**).**

**Manuscript 2**:

**Talent Chipiti, Moganavelli Singh, Mohammed Auwal Ibrahim and Md. Shahidul Islam** **"***In vitro* α-amylase and α-glucosidase inhibitory and cytotoxicity activities of ethanol and aqueous extracts of *Cissus cornifolia* Planch parts", **(** In preparation**).**

**Manuscript 3**:

**Talent Chipiti, Moganavelli Singh, Mohammed Auwal Ibrahim, Neil Anthony Koorbanally and Md. Shahidul Islam** "*In vitro* antioxidant, α-amylase and α-glucosidase inhibition, and cytotoxicity activities of ethanol and aqueous extracts of *Courbonia glauca* (Klotzsch)", **(** In preparation**).**

**PRESENTATIONS**

**Presentation 1**:

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Signed:

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Finally, special thanks to my father Mr. Fungai Chipiti for his inspiration in designing of this project and great help during sample collection.

# Dedication

***This thesis is***

***Dedicated to my dearest parents***

***Mr. Fungai & Mrs. Esnath Chipiti***

## LISTS OF ABBREVIATIONS

| | | |
|---|---|---|
| ADA | – | American Diabetes Association |
| AD | – | Anno Domini |
| AGE | – | Advanced Glycated End-products |
| AO | - | Antioxidants |
| BCE | – | Before the Common Era |
| CVD | - | Cardiovascular disease |
| CSZ | - | Central Statistics Zimbabwe |
| DM | - | Diabetes mellitus |
| DPP-IV | – | Dipeptidyl peptidase-4 |
| DPPH | – | 2,2-Diphenyl-1-Picrylhydrazyl |
| DKA | - | Diabetic ketoacidosis |
| EDTA | – | Ethylenediaminetetracacetic acid |
| EtOH | – | Ethanol |
| GC/MS | – | Gas Chromatography/Mass Spectrometry |
| GLP | – | Glucagon-Like Peptidase |
| GAE | – | Gallic Acid Equivalent |
| IDF | – | International Diabetes Federation |
| MEM | – | Minimum Essential Medium |
| MODY | - | Maturity-onset diabetes of the young |

| | | |
|---|---|---|
| NDM | - | Neonatal diabetes mellitus |
| NPH | - | Neutral Protamine Hagedorn |
| NRF | – | National Research Foundation |
| NIST | – | National Institute of Standards |
| OS or OST | – | Oxidative Stress |
| PKC | – | Protein Kinase C |
| ROS | – | Reactive Oxygen Species |
| T1D | - | Type 1 Diabetes Mellitus |
| T2D / T2DM | – | Type 2 Diabetes/Type 2 Diabetes Mellitus |
| USA CDC | – | United States of America Center for Disease Control |
| UV | – | Ultra Violet |
| S.D. | – | Standard Deviation |
| SPSS | – | Statistical Package for Social Sciences |
| WHO | – | World Health Organization |

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# Abstract

This research was conducted to investigate the *in vitro* anti-oxidative and anti-diabetic effects of medicinal plants, used by traditional healers in Mrewa, Zimbabwe to treat diabetes mellitus and these plants include *Albizia antunesiana, Cissus cornifolia and Courbonia glauca.* These plants were reported by traditional healers to possess therapeutical properties among them antidiabetic potential with less side effects and low toxicity hence candidates for production of safe phyto-formulations in the fight against diabetes mellitus.

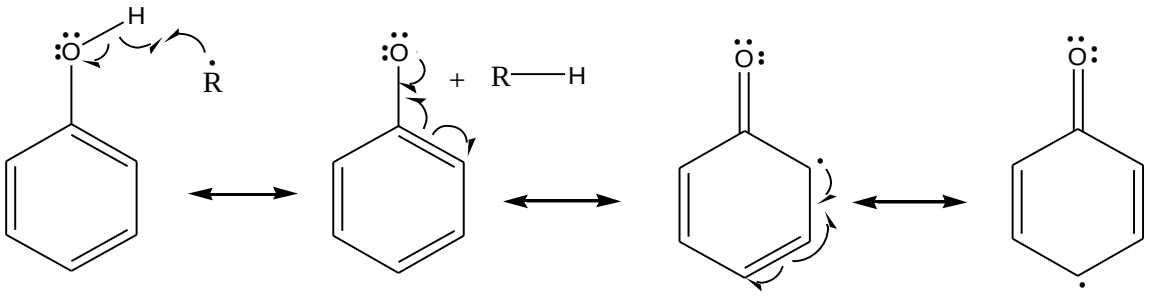
The study was focused on type 2 diabetes mellitus due to its high prevalence such as 90- 95% of all diabetic cases. Since oxidative stress and hyperglycemia have been reported to be key players in the pathogenesis of type 2 diabetes, the main objectives of this study was to evaluate the antioxidative, antihypergycemic and cytotoxic effects of natural products from these plant extracts *in vitro.*

Plant parts (mainly root and leaf) were extracted in ethanol and distilled water after defatting with hexane. A number of models have been used to assay the antioxidant power of the different plants extracts. The results from all the experimental antioxidant models (total reducing power ($Fe^{3+}$-$Fe^{2+}$ ), DPPH, hydroxyl radical and nitric oxide reducing ability) revealed that all the different parts of all plants extracts do possess antioxidant potential in concentration dependent manner to varying extent. However, all the ethanolic root extracts were consistently shown to have exceptionally high antioxidant ability with high phenolics content. However, the antioxidant activity differs from plant to plant in the following order: *Albizia antunesiana, Cissus cornifolia* and *Courbonia glauca* (from most active to the least active in that order).

Furthermore, the effects of plants extracts on carbohydrate hydrolyzing enzyme (α-amylase and α-glucosidase) inhibitions were carried out and *Albizia antunesiana, Cissus cornifolia* and *Courbonia glauca* extracts were shown to possess better α-amylase and α-glucosidase inhibition activities compared to acarbose, a standard drug used for the treatment of diabetes.

In order to ascertain the safety of the extracts, the cytotoxic activities of *Albizia antunesiana, Cissus cornifolia* and *Courbonia glauca* extracts were investigated on human embryonic kidney cells (HEK293). The same trend was observed in all three plants *viz* the ethanolic extracts were

significantly less toxic compared with the aqueous extract and *Albizia antunesiana* ethanolic root extract was the least toxic on the list. Finally, GC/MS analysis was performed to identify the phytochemicals present in the extracts and reviewed the presence of phenolics, coumarins, tripernoids compounds. Compounds with better ability of quenching free radicals (which possess more free hydroxyl groups), such as amyrin and benzene triol were found in the most active extract(s). Better radical scavengers were found to be *Albizia antunesiana* followed by *Cissus cornifolia* then *Courbonia glauca* ethanol root extracts. The identified phenolic compounds possess the ability to donate hydrogen radical or an electron to quench or resultantly neutralize radicals as illustrated below:




However, *in vitro* results alone without *in vivo* test are not enough to give insight about the activity possessed by a particular plant extract. So for the total confirmation of the pharmacological effectiveness of these plant extracts, the *in vivo* tests are required.

Nonetheless, the data presented in this thesis give insight on the value of *Albizia antunesiana, Cissus cornifolia* and *Courbonia glauca* in medicine. This will also mark these plants as potential sources for antioxidant and anti-hypergycemic compounds. The knowledge obtained from this study could be used to lay foundation for further studies on these plants and their extracts and this information will contribute to the field of drug discovery through synthesis of formulations from natural products.

# CHAPTER 1

## GENERAL INTRODUCTION AND LITERATURE REVIEW

## 1.0 Introduction

The global health burden of diabetes mellitus have recently evolved into modern day epidemic and is precisely recognized as a global public health issue which poses major health and socioeconomic challenges (Mbanya *et al.*, 2010). Diabetes mellitus (DM) has turned out to be one of the worst killer disease globally, it's being projected to be the 7$^{th}$ worst killer diseases by 2030 (WHO, 2011). According to International Diabetes Federation (IDF) (IDF Atlas, 5$^{th}$ edition, 2011), approximately 347 million people were diabetic in the year 2010 and it is being projected to reach 552 million by 2030 (Gardner *et al.,* 2011).

Diabetes mellitus is a progressive metabolic disorder with hyperglycemia at its epicenter in all its forms, either due to insulin deficiency or impaired effectiveness of insulin's action, or to a combination of both. The disease is associated with numerous complications which are classified into acute, sub-acute and chronic. Acute complications include hypoglycemia, diabetic hyperosmolar, ketoacidosis and hyperglycemic non-ketotic syndrome, while sub-acute complications are thirst, polyuria, lack of energy, visual blurriness and weight loss (Oyedemi *et al.,* 2009; Calabrese *et al*., 2012). There are various chronic complications of diabetes mellitus which include hypertension, neuropathy, nephropathy, retinopathy and diabetic foot ulcers which could result in amputation, renal failure, heart complications which are a few of the prominent health burdens (Calabrese *et al*., 2012).

The last decade has seen a dramatic escalation in the incidence and prevalence of diabetes worldwide (Mbanya *et al*., 2010). This escalation is more predominant in developing countries this is as a result of adopting westernized diets that contain higher amount of fats and calories compared to conventional diet. Other factors include urbanization and modern technology, sedentary lifestyle etc.

This combination of high calorie diet and less exercise is a recipe for the development of obesity which causes insulin resistance, an important pathogenesis of type 2 diabetes (T2D). Impaired glucose tolerance eventually occurs if insulin resistance persists, this leads to elevated blood glucose level which causes oxidative stress (OS). OS resulting from increased production of ROS (or their inadequate removal) plays a key role in the pathogenesis of late diabetic complications (Brownlee, 2000 and 2001; Calabrese *et al*., 2012). Compelling evidence has

demonstrated the key role played by hyperglycemia in the initiation and the facilitation of OS (Nishikawa *et al*., 2000; Calabrese *et al*., 2012). Hyperglycemia initially increases the production of intracellular ROS and subsequently through, protein kinase C (PKC), advance glycated endproducts (AGE), and sorbitol pathways, later this leads to diabetic complications.

Combating hyperglycemia and OS will go a long way in the treatment and management of many OS mediated disorders including diabetes mellitus. This can be achieved through consuming antioxidants and controlling carbohydrate intake from the diet as well as limiting the caloric intake by inhibiting primary carbohydrate metabolising enzymes, α-glucosidase and α-amylase. The α-glucosidase and α-amylase play a vital role in the regulation of postprandial blood glucose concentration in the human body and its inhibitors, which can subdue postprandial hyperglycemia, are usually used to prevent or treat T2D (Lebovitz and Harold, 1997).

To date anti-diabetic drugs which include metformin, sulfonylureas, thiazolidinediones, insulin, GLP-agonists, meglitanides, DPP-IV inhibitors and α-glucosidase inhibitors have been used and shown significant positive results in the treatment and management of diabetes. However, they have also shown a vast array of side effects namely: nausea, diarrhea, heart failure, drug resistance, cancer, weight gain, hypoglycemia and so on (ADA, 2013).

Alternatively, Natural products, due to their unmatched chemical diversity and biological relevance, have been widely accepted as potential, high-quality chemical pools for the screening of drug candidates. Medicinal plants have been used in primary health care systems, to prevent diseases, maintain health or cure ailments from the dawn of civilization with a number of these plants have been proven to possess various pharmacological activities (Kalemba and Kunicka, 2003).

According to surveys conducted in Africa, more than 5400 plants have been found to have over 16300 uses in medicine and the value of these medicinal plants and their products in the field of drug discovery is quite impressive because a number of clinically active drugs, derived from plant products, have a natural product pharmacophore (Ibrahim *et al.*, 2012). Hence, much attention is given on the screening of medicinal plants with potential bioactive phytochemicals. This has also been reinforced by the fact that there is an increased concern for the safe and non-toxic alternative drugs (Aliyu *et al*., 2012). The shortfalls observed in current conventional drugs

has led the scientific community to investigate plants with the main objective being to uncover natural compounds which can be used to ameliorate diabetes related complications and non-toxic to the patient (Karimi *et al*., 2012).

Hence, this study was conducted to investigate the medicinal properties, specifically the antidiabetic activity of medicinal plants which are claimed to treat diabetes by the Shona speaking people of Mrewa district Zimbabwe. The specific objective was to determine the antioxidant, anti-hyperglycemic and cytotoxicity activity of these medicinal plants *in vitro*.

## 1.1 Literature review

### 1.1.1 Historical background of diabetes mellitus

Diabetes was first described in an Egyptian manuscript from c. 1500 BCE referring "too great emptying of the urine", the first case to be discussed was believed to be type 1 (Poretsky, 2009; Ripoll *et al*., 2011). Physicians in India at the same time observed that urine from diabetic patients attracts ants (termed it “madhumeha” meaning honey urine) (Poretsky, 2009). The term "diabetes" or "to pass through" was first used in 230 BCE by the Greek Apollonius of Memphis. (Poretsky, 2009). It has been reported that diabetes was rare during the ancient times for example during the time of the Roman Empire; according to Galen, only few cases were observed (Poretsky, 2009).

Indian physicians Sushruta and Charaka in 400-500 AD first described T1D and T2D as separate conditions, with type 1 associated with youth and type 2 with being overweight (Poretsky, 2009). Functional treatment was only able to be developed during the beginning of the $20^{th}$ century by Canadians Frederick Banting and Charles Best who discovered insulin in early 1921 and 1922. The invention of long acting NPH (or neutral protamine Hagedorn) insulin followed in 1940s (Baganz *et al*., 1951; Poretsky, 2009).

Diabetes mellitus describes a metabolic disorder of multiple aetiology characterised by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both, due to pancreatic beta cell dysfunction accompanied by the bodies progressive insensitivity to insulin which is termed insulin resistance (Diabetes care, 2003). It results in biochemical and anatomical structural consequences which

have been associated with micro- and macro-vascular complications and morbidity (Levitt, 2008).

Diabetes mellitus have been reported to affect all ages, with T1D mellitus starting to manifest itself from an early age hence termed juvenile onset diabetes and T2D mellitus previously used to start at a later age (adult onset diabetes mellitus). However, due to the dramatic increase of type 2 diabetic cases, it has recently been shown to start at juvenile stages (Brownlee, 2001).

### 1.1.2 Types of diabetes mellitus

Based on the etiological classification, diabetes mellitus can be classified into various categories namely; Type 1, Type 2, Gestational diabetes and some other forms of diabetes (WHO, 2011). However, out of all the subgroups, type 1 and 2 are the most prevalent with type 2 accounting for approximately 90-95% percent of total diabetes cases whereas type 1 is approximately 5-10%.

- Type 1 diabetes mellitus

Type 1 diabetes (T1D) (formerly called juvenile-onset or insulin-dependent diabetes), accounts for approximately 5% to 10% of all people with diabetes. This type is characterized by hyperglycemia when the body is unable to produce insulin due to the autoimmune destruction of pancreatic beta-cells of the islets of Langerhans eventually eliminating insulin production from the body and requires daily administration of insulin to control hyperglycemia. Without insulin, cells cannot absorb sugar (glucose), which they need to produce energy (US CDC, 2008). Currently there is no cure for T1D, so the affected individual will have to solely depend on exogenous insulin injection for survival hence it is called insulin dependent diabetes. Unlike T2D, people with type 1 diabetes generally are not obese and may have diabetic ketoacidosis (DKA) at the initial stage of the disease. The T1D is not preventable with current knowledge (WHO, 2011).

Symptoms of T1D include: excessive excretion of urine (polyuria), thirst (polydipsia), constant hunger (polyphagia), weight loss, vision problem and fatigue. These symptoms usually occur suddenly after the onset of the disease.

- Type 2 diabetes mellitus

Type 2 diabetes (T2D) (formerly called adult-onset or non-insulin-dependent diabetes) can develop at any age, but most commonly becomes apparent during adulthood. But the incidence of T2D in children is rising. T2D accounts for the vast majority of people with diabetes accounting for 90% to 95% (WHO, 1999).

In contrast to T1D, insulin resistance is the main characteristic of T2D. Insulin resistance refers to the body's tissue and cells inability to respond properly to insulin. Resistance develops because of many factors, including genetics, obesity, increasing age, and having high blood sugar for a long time (US CDC, 2008). In T2D, there is an insufficient insulin production accompanied with insulin resistance. In type 2, unlike type 1, patients are not entirely dependent on insulin, since they can still produce insulin; however the body will have lost the ability to sense and utilise it. The development of T2D is caused by a combination of lifestyle and genetic factors (Ripsin, 2009; Risérus, 2009). While some etiological factors such as; diet and obesity, can be controlled or managed while others, such as increasing age, genders, and genetics, are not (Ripsin, 2009). Symptoms may be similar to those of T1D, but are often at lesser magnitude. As a result, the disease may be diagnosed several years after the onset, once complications have already arisen or at an advanced stage. Previously this type of diabetes was seen only in adults but now it is also occurring in children (Vijan, 2010).

Hyperglycemia is a condition when high blood glucose exists in the blood stream and is a common feature of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels (WHO, 2011). Hyperglycemia have been underlined as the main driving forces behind the development T2D associated complications, and sustained hyperglycemia eventually results in a condition commonly referred to as OS. The OS occurs when there is an increased production of reactive oxygen species (ROS) and subsequent decrease in the body’s antioxidant defense mechanism (Kumar *et al.*, 2012). Various mechanisms have been reported to lead the destruction of pancreatic β-cells but OS has been highlighted as one of the main contributing factors (Ibrahim *et al*., 2012).

- Gestational Diabetes Mellitus

Gestational diabetes (or gestational diabetes mellitus, GDM) is a condition when women who have previously never been diagnosed with diabetes exhibit high blood glucose levels during pregnancy. Gestational diabetes is at present affecting approximately 3-10% of pregnant women, however it depends on the population studied (ADA, 2013).

This is possibly a pregnancy-related aspect such as the presence of human placental lactogen that interferes with susceptible insulin receptors. The exact mechanisms underlying the cause of GDM are still elusive (WHO, 2011; ADA, 2013).

The major cause of GDM has been reported to be increased insulin resistance. During pregnancy fluctuations in hormonal levels and other causes are thought to obstruct the action of insulin. The interference possibly occurs at the level of the cell signaling pathway behind the insulin receptor (Carr and Gabbe, 1998). Symptoms of gestational diabetes are similar to those of T2D. Gestational diabetes is most often diagnosed through prenatal screening, rather than reported symptoms (Carr and Gabbe, 1998; WHO, 2011).

Some second generation oral glycaemic agents such as Glyburide have been shown to be safe in the treatment of GDM. The GDM usually disappears after pregnancy but women with GDM and their children are at an increased risk of developing T2D later in life. Approximately half of the women with a history of GDM develop T2D within five to ten years after delivery (IDF Atlas, 5th edition, 2011; Carr and Gabbe, 1998).

- Other forms of diabetes or prediabetes

There are quite number of other forms of diabetes that exist (WHO, 2011). Other forms of diabetes include those caused by or as a result of:

- Genetic error or defects of the beta cell, the part of the pancreas that synthesizes insulin— these include maturity-onset diabetes of the young (MODY) or neonatal diabetes mellitus (NDM)
- genetic defects in the action of insulin, this will cause the body unable to control blood glucose levels, as seen in leprechaunism and the Rabson-Mendenhall syndrome

- other diseases of the pancreas or other conditions that results in the damage of the pancreas, include pancreatitis and cystic fibrosis
- excess amounts of certain hormones resulting from some medical conditions—such as cortisol in Cushing's syndrome—that work against the action of insulin
- medications that reduce insulin action, such as glucocorticoids, or chemicals that destroy beta-cells
- infections, such as congenital rubella and cytomegalovirus
- rare immune-mediated disorders, such as stiff-man syndrome, an autoimmune disease of the central nervous system
- genetic syndromes associated with diabetes, such as Down syndrome and Prader-Willi syndrome

### 1.1.3 Type 2 diabetes mellitus and antioxidative stress

- Oxidative Stress (OS)

OS is a physiological condition where there is presence of products called free radicals, which are oxygen centered free radicals and molecules having an unpaired electron and reactive oxygen species (ROS) or oxidants. This can result either from an excess production of ROS or from the inactivation of the antioxidants (AO), thus shifting the ROS/AO equilibrium towards stress (Ibrahim *et al.*, 2012).

These molecules originate from normal physiological processes of aerobic organisms and if not quenched by a cascade of antioxidant systems, they become deleterious. All aerobic organisms require oxygen for energy production in the form of ATP, which is achieved by metabolising variety of energy sources like fatty acids, glucose, lactate, ketones, and amino acids via producing ATP, NADH and $FADH_2$. These cofactors donate electrons to the electron transport chain which will generate chemical energy in the form of ATP. This process is normally terminated by oxygen which is the last electron acceptor. During this stage Dioxygen accepts electrons to make it Superoxide anion radical, an unstable ROS. So this makes superoxide radical to be the "primary" ROS, it consequently generates "secondary" ROS, either directly or indirectly by a variety of enzymes or metal catalyzed processes (Wiernsperger, 2003).

Plants and animals have an enzyme called superoxide dismutase (SOD) which catalyses the conversion of superoxide to hydrogen peroxide, a weak ROS (Wiernsperger, 2003). Hydrogen peroxides cross the membranes and reacts with superoxide by inactivating the enzymes through oxidations of their thiol groups. According to Haber-Weiss and Fenton reactions, hydrogen peroxide is converted to hydroxyl radicals in the presence of transition metals. Other ROS formed in living systems are singlet oxygen and peroxyl radical (ROO•). Singlet oxygen is generated in neutrophil by the reaction between hypochlorous acid (HOCl) and hydrogen peroxide. Peroxyl radical is generated from the reaction between hydroxyl radical and singlet oxygen with organic molecule like lipid.

- Normal functions of ROS in physiological system

The body requires minute quantity of ROS to facilitate its normal physiological processes like gene expression, cellular growth and defense against infection and to also act as the stimulating agents for biochemical processes within the cell (Wiernsperger, 2003). They affect this through oxidation of transcription factors such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) active sites, leading to gene expression which results in cell growth and development. ROS are involved in normal function of the immune system (T cell differentiation, kill pathogens and apoptosis), development (cell growth and proliferation, fusion of sperm and growth, synthesis of thyroxine) and activation of signal transduction pathways if present in normal concentrations.

- Overproduction of ROS

If ROS is in excess (high concentration), it becomes deleterious by compromising a vast array of biochemical pathways and by doing so it promotes the damage to macromolecules such as nucleic acids (DNA), lipids, proteins and might have an active role in the pathophysiology of several diseases (Wiernsperger, 2003).

Cell membranes are more vulnerable to ROS damage due to their high concentration of lipids and damage to lipids is referred to as lipid peroxidation, this damage occur as multiple stage process which eventually leads to cross linking of membrane proteins, change in membrane fluidity and formation of lipid-protein, lipid-DNA adduct which may be detrimental to the functioning of the cell (Markesbery, 1997; Halliwell, 2000; Zhang *et al.*, 2006)

On the other hand, proteins form the main pool of cell constituents and ROS inflict damage to proteins through a range of reactions which causes oxidation and change their tertiary structure leading to proteolytic degradation, protein-protein cross linkages and fragmentation resulting in the loss of the biological activities and decreased cellular functions such as energy production (Cabiscol *et al.*, 2000). ROS interact with DNA molecules altering its structure and function through modification of DNA bases, single and double strand DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system. These have a detrimental effect of causing cell death, mutagenesis, carcinogenesis and ageing.

- Antioxidants vs ROS

Antioxidants are a group of molecules and substances, which inhibit damages induced by the oxidants by reacting with ROS and converting them into neutral harmless compounds. All aerobic organisms have acquired their own antioxidant defenses system to quench the excess free radicals, however when OS is prevailing exogenous antioxidant supplementation becomes unavoidable (Wiernsperger, 2003; Zhang *et al.*, 2006). The ROS are continuously generated from various biochemical processes which lead, to accumulation of these compounds in excess amounts, in the body overtime, hence cell have evolved an antioxidant system to keep the concentration of ROS in check. This is through antioxidant defenses and repair mechanisms. There are various defense mechanisms present in the body, however the one which acts directly by removing the oxidant are considered much more effective and offer maximum protection to cells. Three mechanisms have been proposed that antioxidants normally operate; i) preventing: keeping ROS at a minimum concentration possible, ii) interception: quenching and scavenging reactive species either by using catalytic and noncatalytic molecules and (c) repair: repairing damaged target molecules (Helmut, 1997)

- Antioxidant defense systems

Antioxidant defense systems operate in two main forms, the enzyme mediated and the non-enzyme mediated antioxidant defense. Enzymatic antioxidants present in the body include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Wiernsperger, 2003).

SOD protects cells from superoxide toxicity by catalysing the conversion and dismutation of superoxide into oxygen and hydrogen peroxide (Wiernsperger, 2003).

Catalase is mostly abundant and located in the peroxisomes, since peroxisomes are involved in fatty acid oxidation and hydrogen peroxide is one of the end products of fatty acid peroxidation, so catalase prevents the toxic effect caused by hydrogen peroxide to the cell. This is achieved by catalysing conversion of hydrogen peroxide to water and molecular oxygen (Wiernsperger, 2003). GPx is selenium containing antioxidant enzyme that catalyses the reduction of various hydroperoxides in the presence of reduced glutathione (Wiernsperger, 2003).

Various low molecular weight non-enzymic molecules also act as the body's antioxidant defense line, this include glutathione (GSH), α-tocopherol, ascorbate , bilirubin (Wiernsperger, 2003; Kunwar, 2009) and some excretory compounds such as bilirubin from heme, urate from uric acid and also a lot of proteins such as lothionein, ceruloplasmin, ferritin, lactoferrin, transferrin, metallothionein (Wiernsperger, 2003).

The body is also equipped with the ability to repair ROS mediated DNA damage. These mechanisms are specific to particular damages and they include photoreactivation, base excision and nucleotide excision repair (Halliwell and Gutteridge, 1989; Wiernsperger, 2003).

In order to counteract the effects of OS, in some cases, exogenous antioxidant supplements are used to restore the bodys homeostasis state. These supplementations come in form of fruits, legumes, vegetables, tea, herbs and wine. Their antioxidant ability is attributed to the presence of secondary metabolites but mostly polyphenols are capable of donating a hydrogen radical or an electron to quench radicals as the resultant aromatic radicals are able to be stabilized by delocalization of the resultant radical on the benzene ring (Wiernsperger, 2003).

- Synthetic antioxidants

Apart from the natural antioxidants, there have been a wide range of synthetic antioxidants incorporated in cosmetics, pharmaceuticals and food products (Wiernsperger, 2003). These include synthetic antioxidative agents, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tertiobutylhydroxytoluene (Kunwar, 2009).

Thus, the use of antioxidants to counteract the effects of OS in OS-related chronic metabolic disorders is an established protocol. T2D is one of such OS related chronic metabolic disorders. In T2D, excessive levels of glucose reaching the mitochondria lead to an overdrive of the electron transport chain, resulting in overproduction of superoxide anions normally scavenged by mitochondrial SOD. When it fails OS develops and it has been recently proposed that this mechanism is responsible for the activation of all major pathways underlying the different components of vascular diabetic complications (Wiernsperger, 2003).

Furthermore, hyperglycemia generates and can induce OS by various mechanisms among them is: glycation (increased formation of advanced glycation end products (AGEs), PKC activation, sorbitol pathway, activation of the renin–angiotensin system (RAS) and autoxidation of glucose. These mechanisms have been reported to be the major contributors of diabetic vascular complications (Wiernsperger, 2003; Yamagishi and Imaizumi, 2005), mitochondrial coupling of NOS by hyperglycemia has also been implicated to be a major contributor of development of OS (Brodsky *et al.,* 2002).

- Autoxidation of glucose

Autoxidation of glucose is one potential source of oxygen-derived free radicals (OFRs) in diabetes, autoxidation of glucose and other monosaccharides is a process which involves chain reactions catalysed by transition metals in the presence of excess glucose, eventually it leads to the production of free radicals also called reactive oxygen species (ROS) (Wiernsperger, 2003; Brodsky *et al.,* 2002; Zhang *et al.,* 2006). Mono saccharides can enolize and thereby reduce molecular oxygen under physiological conditions, yielding-ketoaldehydes, $H_2O_2$ and free radical intermediates which upsets the ROS production and their subsequent elimination by the body's antioxidant system leading to OS (Simon and Rodgers, 1987). The continuous occurrence of this process contribute to elevated levels of peroxides and oxides and if unquenched by a cascade of antioxidant system will became deleterious by further promoting existence of OS. Glucose can oxidize and generates ROS, hydrogen peroxide, and reactive ketoaldehydes. These later compounds largely participate in the synthesis of glycated proteins, which are themselves a source of OFRs (Simon and Rodgers, 1987).

- Glycation

It is basically a non-enzymatic reaction of reducing sugars and amino groups of proteins, lipids and nucleic acids which causes aging of macromolecules; this process has been known to be accelerated at an alarming rate under hyperglycemic and/or OS conditions (Yamagishi *et al.,* 2012).

According to Dyer *et al*. (1991), Stern *et al.* (2002), and Takeuchi and Yamagishi (2008), excess reducing sugars (glucose) under hyperglycemic conditions reacts non-enzymically with proteins to form compounds and molecules called Amadori products which undergo further rearrangements overtime to form cross-linked irreversible senescent macro protein derivatives called Advanced Glycated End products (AGEs).

Formation of AGEs under diabetic condition is accelerated at an alarming rate, these AGEs have been implicated in the development of OS through their reaction with Receptors of Advanced Glycated End products (RAGEs) and consequently induce inflammatory and thrombogenic reactions, thus playing a significant role in the development and progression of vascular complications in diabetes (Yamagishi *et al.*, 2008; Takenaka *et al.,* 2006). Additionally, AGEs are reported to promote and up-regulate RAGE expression in numerous cell types and activate of transcriptional factor nuclear factor-κB (NF-κB) (Yamagishi *et al.*, 2008).

Henceforth, it is possible that the AGE–RAGE-induced OS generation additional facilitates the creation and buildup of AGEs and subsequent RAGE overexpression in diabetes (Brownlee, 2001; Yamagishi, 2005).

### 1.1.4 Complications of type 2 diabetes mellitus

Diabetes is also associated with a wide range of degenerative late complications involving various organs, which progress over many years. Generally, deleterious effects of hyperglycemia are classified into macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Fowler, 2007).

These can be further categorized into three more groups which are acute, sub-acute and chronic complications. Acute complications includes hypoglycemia (excessively low blood glucose), diabetic ketoacidosis (accumulation and production of keto acids which are deleterious), hyperosmolar (severe dehydration due to high blood glucose) and hyperglycemic non-ketotic syndrome (Knentz and Nattras, 1991) while sub-acute complications are thirst, polyuria, lack of energy, visual blurriness and weight loss (Kumar and Clark, 2002). The chronic complications of diabetes mellitus include hypertension (high blood pressure), neuropathy( disease of the nervous system), nephropathy(kidney complications), retinopathy (eyes disease which might lead to blindness) and diabetic foot ulcers which could result to amputation, renal failure, heart complications which are a few of the prominent health burdens.

- Microvascular complications of diabetes

Several underlying mechanisms have been suggested to be responsible for the onset and development of diabetic microvascular complications namely diabetic retinopathy, nephropathy and neuropathy.

Diabetic retinopathy is thought to be the most prevalent of all known vascular complications and the risk of developing retinopathy depends on the duration and severity of hyperglycemia (Fowler, 2008). Proposed pathological mechanisms in the development of retinopathy include the conversion of glucose into sorbitol in the polyol pathway which has a consequence of increasing the osmotic stress, cell are injured by the accumulation of glycoproteins from the formation of AGEs. OS environment have also been thought to facilitate microvascular complications through interaction of free radical and different biomolecules (Kunisaki *et al.*, 1995; Fowler, 2008).

In diabetic nephropathy, several pathological changes occurs which includes increased glomerular basement membrane thickness, micro-aneurysm formation, mesangial nodule formation (Kimmelsteil-Wilson bodies), and other changes. The mechanisms responsible for this complication involve some or all of the same mechanisms as diabetic retinopathy (Fowler, 2008).

American Diabetes Association (ADA) 2007 recognizes diabetic neuropathy as the "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes" (Fowler, 2008).

The extend of the injury inflicted to the peripheral nerves due to hyperglycemia is not fully understood but is proposed to be directly linked to mechanisms such as accumulation of polyol, damage from AGEs, and prolonged OS conditions. In diabetics, peripheral nerve damage has been found to occur in various forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations have been reported to result from foot ulceration or injury, which can result from diabetic neuropathy (Fowler, 2008).

- Macrovascular complications of diabetes

According to Fowler (2008), the central theme in macrovascular complications is the process of atherosclerosis, which results in accumulation of substances and narrowing of arterial walls throughout the body. Atherosclerosis is as a result of chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system which might have originated from microvascular damage as a result of prolonged hyperglycemia.

To counteract the endothelial injury and inflammation, oxidized lipids accumulate followed by monocytes which later become phagocytes and macrophages. This attracts T-lymphocytes that induce smooth muscles to proliferate and develop into collagen. Besides atheroma formation, there is also increased adhesion hyper coagulation of blood cells and various substances during T2D. Impaired nitric oxide generation and increased free radical formation in platelets, as well as altered calcium regulation, may promote platelet aggregation.

Diabetes has been reported to increase the risks of developing cardiovascular disease (CVD) however the exact underlying mechanism(s) for the development of atherosclerosis is poorly understood, nevertheless, scientific evidences have provided a profound link between these two diabetes associated complications. This is also supported by the fact that CVD is the primary cause of death in diabetics (Laing *et al.*, 2003; Paterson *et a*l., 2007; Fowler, 2008).

### 1.1.5 Treatment and management of diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder, at present there is no known cure apart from very specific situations, management is mainly focused on regulating the blood sugar levels as close to the normal level as possible ("euglycemia") without causing hypoglycemia. This is achieved by following a strict well regulated program over a long time. Alternatively to achieve ("euglycemia") is through consuming a well regulated diet, exercise, and use of appropriate medications consistently.

The accumulative nature of DM as well as late complications calls for the constant reassessment of the effectiveness of the treatment and to check if adjustment of the therapeutical is required (Deutschländer *et al.,* 2009). This will result in prohibitive cost and ultimately unavailability of drugs and treatment to low income and underprivileged communities particularly in developing countries.

- Diet and exercise in the management and treatment of diabetes mellitus

Lifestyle interventions such as proper regulation of diet and exercise have proven to be much effective before the onset of diabetes. Some previous studies (Markovic *et al.*, 1998; Maggio and Pi-Sunyer, 1998) clearly demonstrated the benefit of a healthy diet, regular exercise, and weight loss in individuals already diagnosed with diabetes which include regained insulin sensitivity and decrease fasting glucose levels. A combined regiment of a strictly controlled diet and regular exercise had been shown to help weight loss and prevent weight regain (Folwer, 2007).
Furthermore, Colberg *et al*., (2010) reported that exercises have been regarded to be one of the cornerstones in the treatment and management of T2D due to its ability to regulate blood sugar levels, with minimal undesired side effects.

- Current conventional drugs for diabetes mellitus

Currently anti-diabetes drugs are available as generics of oral hypoglycaemic agents (ADA, 2013). These include; Sulfonylureas, Biguanides, Thiazolidinediones Alpha-glucosidase inhibitors, Meglitinides - nateglinide

- **Insulin Sensitizers**

This class of oral antidiabetic agents acts by increasing insulin sensitivity of cells and tissues.

- **Metformin**

Metformin is a biguanide and an analog of galegine, an alkaloid first reported by French diabetologist Jean Sterne, which possesses anti-hyperglycemic properties and originates from the French lilac or goat's rue (*Galega officinalis*), a plant used in folk medicine for several centuries (Witters, 2001; Bailey and Day, 2004). Metformin works by suppressing glucose production by the liver and now believed to be the most widely prescribed antidiabetic drug in the world (Bailey and Day, 2004). Its side effects includes gastrointestinal tract disturbance (nausea, diarrhea, vomiting) (Kahn *et al.*, 2006), lactic acidosis (Fimognari *et al.*, 2006) and so on. Other biguanides include; Phenformin, Buformin.

- **Thiazolidinediones (Glitaziones)**

This class of drug includes: rosiglitazone, pioglitazone, troglitazone. They work via binding to PPARγ, a nuclear regulatory protein that facilitates the transcription of glucose and fat metabolism regulating genes. The PPARs acts on peroxysome proliferator responsive elements (PPRE), these activates insulin sensitive genes hence increased glucose uptake by cells (EMA, 2009; FDA, 2011) however they have been reported to cause urinary bladder cancer, and macular edema and heart failure (FDA, 2011).

- **Insulin Secretagogues**

These classes of oral antidiabetic agents function by stimulating insulin release from pancreatic β cells (Neumiller and Setter, 2006).

- **Sulfonylureas**

This class of drug includes glyburide, glimepiride, and glipizide. They work through stimulating insulin release by pancreatic beta cells via inhibiting the $K^+_{ATP}$ channel (Agabegi and Steven, 2008). Sulfonylureas bind to an ATP-dependent $K^+$ ($K_{ATP}$) channel on the cell membrane of pancreatic beta cells. This inhibits a tonic, hyperpolarizing efflux of potassium, thus causing the electric potential over the membrane to become more positive. This depolarization opens voltage-gated $Ca^{2+}$ channels (Neumiller and Setter, 2006).

The rise in intracellular calcium leads to increased fusion of insulin granulae with the cell membrane, and therefore increased secretion of (pro) insulin. However, the side effects of sulfonylureas include: hypoglycaemia, liver and kidney damage, cardiovascular damage, abdominal upset, headache and hypersensitivity reactions (Patel *et al.*, 2008)

- **Meglitanides (**short-acting insulin secretagogues)

This class of drugs includes repaglinide, nateglinide. They act by inducing the production of insulin by pancreastic beta cells. They act on the similar potassium channels as sulfonylureas, but at a different binding site. Closing the potassium channel and the opening of the calcium channel will facilitates the secretion of insulin by the pancreatic beta cells (Rendell, 2004). Most insulin secretagogues causes hypoglycaemia and weight gain (Agabegi and Steven, 2008; Rendell, 2004)

- **Alpha glucosidase inhibitors**

This class of drugs includes acarbose (Precose/Glucobay), miglitol (Glyset), and voglibose. Alpha-glucosidase inhibitors (AGIs) are classified as diabetic pills than hypoglycaemic agents since neither possesses a direct effect on insulin secretion or sensitivity. They act by binding competitively to enzymes that degrade complex carbohydrates in intestines, preventing metabolism of complex sugars (polysaccharides) into monosaccharides e.g. glucose. Hence, they reduce postprandial blood glucose excursions. This will results in the low blood glucose to be matched more effectively by an impaired insulin response or sensitivity (Germino, 2011).

AGI use has been associated with gastrointestinal disturbances (flatulence and bloating) (Inzucchi, 1998)

- **Peptide analogs (incretin-based therapies)**

Basically they can be further classified as insulin secretagogues which are glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (glucose-dependent insulinotropic peptide, GIP) and they are both deactivated by the enzyme dipeptidyl peptidase-4(DPP-4) (Briones, 2006). They interferes with the homeostasis of glucose via stimulating the glucose dependent pancreatic insulin secretion by suppressing the secretion of glucagon (glucose dependently), by also

delaying gastric emptying, increases satiety, and decreasing food intake (Williams *et al.*, 1996 and Nauck *et al.*, 1997)

- **GLP-1 Receptor Agonists**

This class of drugs includes Exenatide (Exendin-4/Byetta), Liraglutide (Victoza), Taspoglutide. Glucagon-like peptide (GLP) agonists bind to a membrane GLP receptor. As a consequence, insulin release from the pancreatic beta cells is increased (Amori *et al.*, 2007). They are alleged to cause decrease in gastric motility which results in nausea and thought to be responsible for the weight lose observed in patients using this drug. The side effects includes acute pancreatitis, renal injury, minor hypoglycemia, and/or nausea (Davidson *et al.*, 2010)

- **DPP-4 Inhibitors**

This class of drugs includes vildagliptin (Galvus), sitagliptin (Januvia), saxagliptin (Onglyza), linagliptin (Tradjenta), allogliptin, septagliptin. The oral DPP-4 inhibitors function by inhibiting the metabolism of GLP-1 and GIP which results in decreasing the half-life and blood levels of these endogenous incretins. However, they pose side effects like nasophryngitis, urinary tract infection, headache and acute pancreatitis (Amori *et al.*, 2007; Germino, 2011).

- **Amylin analogues**

There is only one analogue of amylin such as pramlintide (Symlin). Amylin or Islet Amyloid Polypeptide (IAPP) which is co-secreted with insulin in miniature volumes by the beta cells of the pancreas. Amylin plays a crucial role in glycemic regulation through slowing gastric the emptying gastric and promoting satiety, thereby decreasing post-prandial spikes in blood glucose levels (Higham *et al.*, 2000). Amylin agonist analogues slow gastric emptying and suppress glucagon. Side effects include nausea.

On the contrary, current evidence supports the fact that the available diabetes treatment regiments can improve the body's glucose control through a variety of mechanisms, however all the available medication has indicated to have severe side effects varying with individuals. Therefore, the scientific community is still burdened with the tasks of further investigating

alternative sources, with the ultimate goal of developing compound(s) which will ameliorate diabetes related symptoms and/or cure for diabetes without concurrently causing adverse effects.

Most notably, the OS and hyperglycemia are the central themes in the pathogenesis of diabetes mellitus (Calabrese *et al.*, 2012). Given the rate, at which people are consuming high calorie diets, the control or proper management of postprandial hypergygycaemia or blood glucose spiking after a meal is mandatory and of the utmost importance in preventing and treating diabetes (Kim *et al.*, 2005).

The most effective way to regulate postprandial plasma glucose concentration is with medication in combination with dietary restriction and an exercise program (Goke and Herrmann-Rinke, 1998). One of such approach involves decreasing the absorption of glucose from the gut; this is achieved by inhibiting carbohydrate digesting enzymes (e.g., α-amylase and α-glucosidase) (Toeller, 1994; Kim *et al.*, 2005). This will result in the low blood glucose to be matched more effectively by an impaired insulin response or sensitivity (Germino, 2011), in diabetic and in prediabetes cases. A range of synthetic conventional alpha glucosidase inhibitors (AGI) exist and they include; acarbose (Precose/Glucobay), miglitol (Glyset), voglibose. However, AGI has been associated with gastrointestinal disturbances (flatulence and bloating) (Inzucchi, 1998), this has prompted the search for alternate AGI from safer and natural sources. Alpha glucosidase inhibitions have been one of the mechanisms of action that several plants and mushrooms were reported to possess this activity (Matsuur *et al*., 2002; Sama *et al.*, 2012). This also points out that some medicinal plants may have bioactive compounds which are analogs of drugs such as acarbose.

The direct link which exist between hyperglycemia and OS, necessitate the scientific community to search for compounds which can inhibit carbohydrates digesting enzymes and the oxidation of oxidizable molecules in a chain reaction and such compounds could consequently be vital in the therapy and prevention of diabetic complications (Wright *et al*., 2006). Unfortunately, although conventional α-glucosidase inhibitors and synthetic antioxidative agents, exhibit antidiabetic activities, they induce liver damage, carcinogenesis and gastrointenal tract disturbance (Saito *et al*., 2003; Subhasree *et al*., 2009; Inzucchi, 1998).

Therefore, there is a need for the development and utilization of more effective alternative drug sources of natural origin (Djeridane *et al*., 2010). Undesirable effects caused by the use of synthetic antidiabetic drugs which include stomach upsets, drug resistance just to name a few, prompted many researchers now to focus on compounds of natural origins and mostly from plants that demonstrated to have antidiabetic potential with less or no side effects and these plants would be good candidates for the production of good phyto-formulations in the fight against diabetes mellitus (Shai *et al.*, 2008).

### 1.1.6 Plants as alternative source for novel antidiabetic compounds.

Plants had been used by mankind since time immemorial for foods, cloths and also as a source of medicine. A lot of derivatives with plants origin are well documented dating back to centuries worldwide. African traditional medicine is regarded the most aged and the most varied of all traditional medicinal systems (Van Wyk, 2008).

Given that Africa, specifically southern Africa do possess quite a rich diversity of plants; with statistics pointing out that approximately about 25% of the world's higher plants are found in the Sub-Saharan Africa (Van Wyk, 2008). Furthermore, it's a well-known fact that usage of plants for medicinal purpose is a practice which dated back to ancient times, especially in the African society. Van Wyk (2008) also reported that in Africa, there are approximately more than 5000 medicinal plants that had been reported to have over 16,300 medicinal uses.

This practice remains to be existent in the developing nations and it is on this foundation that researchers keep on working on medicinal plants in order to produce/develop the best medicines for physiological uses (Musa *et al.*, 2011). Due to these concerns, in recent years, many studies are aimed in searching for alternative safer medication for example the screening of medicinal plants and plant products for pharmacologically active compounds.

Patel (2012) reported that a lot of plants and herbs have been used by many cultures as dietary adjuvant to treat a number of ailments without actual knowledge of activity and constituents present in them. Recently, there has been a rise in adoption and recommendation of medicinal foods, even when their biologically active compounds are unknown, due to their availability, safety and effectiveness (Patel, 2012; Dewanjee *et al*., 2009).

This has prompted World Health Organization (WHO) to encourage the intensive investigation and screening of traditional plant treatments for diabetes and those that show impressive antidiabetic activity with minimal or no side effects are to be considered as excellent candidates for alternative oral antidiabetic therapy (Patel, 2012).

According to WHO 2011, 70-95% of the world's population, particularly from developing countries, rely heavily on plant-derived medicines for their healthcare, in some worst cases traditional medicine is not only approved and popular but it is the only healthcare system available in remote areas (Mabona and Van Vuuren, 2013).

In Zimbabwe, it is reported that approximately 5000 plant species are known and more than 500 (~10%) of them have known medicinal values (Maroyi, 2013). A research conducted by Maroyi (2013) indicated that the knowledge of the importance of medicinal plants is enormous. Fakim (2006) reported that traditional healers and herbalists do not possess the knowledge to explain the mechanism of actions of their medicine and this makes the practice inferior and associated to witchcraft.

On the contrary, scientific researchers have a better understanding of how the body functions, which put them at a better position to comprehend the healing powers of plants and their potential as multi-functional chemical entities for treating complicated health conditions and this makes the use of plant-derived medicines becoming much scientifically popular recently.
Approximately 800 plants have been reported to have anti-diabetic activities which vary from alpha glucosidase inhibition, glucose transport interference, insulin secretion stimulation, enhancing glucose tolerance and stimulation of insulin sensitivity (Patel, 2012). This mechanism of action displayed by different plants has been strongly tied to phytochemicals present in the plant extracts; alkaloids, flavonoids, phenolics, glycosides, methylated sugars, polysaccharides, saponins to name a few (Patel, 2012).

The use of medicinal plants have been adopted since ancient times to treat a lot of ailments, some of the plants and plant derived materials shown impressive potential to influence the discovery of some current conventional medicines. Natural products have influence the discovery of new

active drugs or analogs which have natural product pharmacophore e.g. metformin an analog of galegine which was extracted from plant *galega offinalis* (Jarald *et al.*, 2008; Patel, 2012).

Besides glucose lowering effects, a number of plants have been reported to have the ability to facilitate beta cell regeneration, antioxidant activity, cholesterol lowering activity and restoration of normal glycogen levels. Unfortunately, some reports indicated the presence of some potential toxic and carcinogenic (Fennell *et al*., 2004) agents in some of these plants making them unsuitable for therapeutic applications. It is therefore of the utmost important to intensively investigate the potential cytotoxic activity in order to validate safety for the continued use of medicinal plants. It has been documented that some plants extracts do have bioactivity however it is cancelled by their cytotoxicity; hence such scenarios need to be evaluated to assess the overall efficacy of the plant extract. However, in Zimbabwe, the research data on anti-diabetic medicinal plants are poorly documented henceforth this study aimed to fill in that gap and furthermore reporting on different mechanisms of actions of medicinal plants used for the treatment and management of T2D. The plants were mainly collected from the Mrewa district of Zimbabwe near to Harare (Fig. 1.1) and mainly investigated the antioxidant, carbohydrate digesting enzymes inhibitory and cytotoxic activities of 3 different medicinal plants usually used by traditional healers for the treatment of diabetes.

### 1.1.7 Plants under study

- Description, brief systematics and geographical extension of the plants under study

Map of Zimbabwe showing Mrewa District

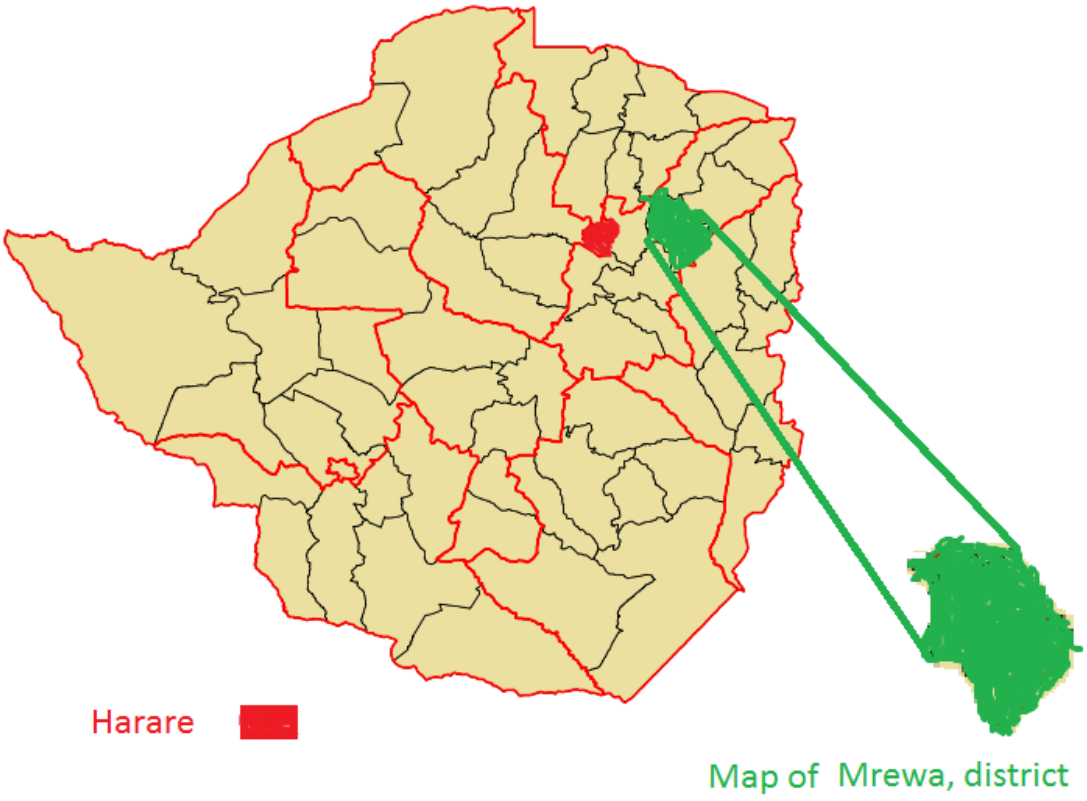



**Fig. 1.1** Zimbabwe Province - and District- map, Showing capital Harare in red and Mrewa district in green (Central Statistics Zimbabwe, 2012).

Mrewa is a town in Zimbabwe, which is 75 kilometers north-east of the capital Harare, along the Nyamapanda road towards Mozambique border. It is in the province/state/region of Mashonaland East with a population of ~195 000, located on coordinates Latitude: 17° 39' 13" S and Longitude: 31° 48' 30" E and an altitude of 1 363 m (CSZ, 2012).

This study was conducted to investigate five medicinal plants used by Shona people of Mrewa district in the eastern part of Zimbabwe to treat diabetes. Through personal communications, the selected plants were mentioned by at least three or more traditional healers or herbalists to be

used to treat amongst other diseases and ailments, diabetic mellitus. All the selected plants were from different families and genus and they are: *Albizia antunesiana, Cissus cornifolia* and *Courbonia glauca* .

- ***Albizia antunesiana* harms** (Fabaceae).

**Fig. 1.2** Picture of *Albizia antunesisana* harms, taken from Cleveland Dam, Mrewa, Zimbabwe.

*Albizia antunesiana* Harms (Fabaceae), commonly called purple-leaved albizia, is indigenous to Zimbabwe. Locally, it is called "Muriranyenze" and "Umnonjwana" by the Shona and Ndebele-speaking Zimbabweans, respectively (Hyde *et al.*, 2013). It is a small to medium-sized tree with pinnated leaves which are subdivided into leaflets and only the young saplings have distinctly purple leaves which turns paler underneath when matured (Hyde *et al.*, 2013). The tree is mostly distributed in southern Africa (Angola, Botswana, Namibia, DRC, Tanzania, Malawi, Mozambique, Zambia and Zimbabwe). In Zimbabwe, it is distributed countrywide (Drummond, 1981). Albizia belongs to the Fabaceae or Leguminosae family and encloses 150 species of which 48 are found in Africa, 35 in America and 35 in Asia and is a genus commonly known as the legume, pea, or bean families, which are a large and economically important family of flowering plants (Stevens, 2001).

Based on a survey contacted in Mrewa and Mutoko districts in Zimbabwe, personal communications reviewed that; the leaves and roots of this plant are expansively used by various traditional healers, herbalists and general people for curing several metabolic and non-metabolic

disorders and diseases such as sore eyes, cuts, ulcers, sore throat, tonsillitis, tuberculosis, gonorrhea, diabetes and cardiac problems. Gelfand et al. (1985) also substantiate this claimed reporting that this plant is also used by traditional healers in other regions of Zimbabwe to treat other ailments such as: cuts, ulcers, sore throat and tonsillitis.

Randriamampianina *et al*. (2013) reported that most plants in the genius *Albizia* contain bioactive compounds like; oleanane-type triterpenoid, saponins, furofuran-type lignan glycosides, macrocyclic spermine alkaloids, flavonol glycosides (Kang, 2000), phenolic glycosides and pyridoxine derivatives known as ginkgotoxins. There haven't been any scientific reports so far to substantiate the traditional use of albizia as an antidiabetic agent.

- ***Cissus cornifolia* (Baker) Planch (**Vitaceae)

**Fig. 1.3** Picture of *Cissus cornifolia* plants, taken from Chivake River, Mrewa, Zimbabwe

*Cissus cornifolia* (Baker) Planch (Vitaceae) commonly called the "Ivy grape" is indigenous to Zimbabwe. Locally, it is called ''Mudzambiringa'' and ''Idebelebe'' by the Shona and Ndebele-speaking Zimbabweans respectively. *Cissus cornifolia* is a species in the genus Cissus which have approximately 350 species of woody climber in the grape family (Vitaceae), the plant is an annual sub erect herb with height of about 1.5 m from the permanent woody root base. It has a cosmopolitan distribution, though the majorities of this family are confined to the tropics.

The survey we conducted in Mrewa district in Zimbabwe in 2012 based on personal communications with traditional healers and herbalists reviewed that**,** the plant (*Cissus cornifolia*) has wide array of uses in African traditional medicine amongst them as an antidiabetic agent. It was also pointed out that it is used by the Shona speaking people as a remedy for gonorrhea when the leaf, bark or root extract taken with native natron while the leaf-sap is used among the Tanganyika as a sedative in cases of mental derangement. The root-decoction is also used for malaria, septic tonsil, diabetes, cardiac problems and pharyngitis (Burkill, 2000).

Musa *et al*. (2008) reported the methanolic leaf extract of *C. cornifolia* to possess some neuropharmacological activity *in vivo* using mice and this was further supported by the presence of alkaloids, flavonoids, stilbenoids, saponins, steroids and tannins. Jimoh *et al*. (2013) also reported the presence of alkaloid, flavonoids, saponins, steroid, terperiods and tannins in methanolic leaf extract of *C. cornifolia* and they also reported its hypogycemic activity on normoglycemic rats. Further investigations indicated its ability to lower glucose in alloxan-induced diabetic rats. Until now, there is no report on the antioxidant, alpha glucosidase inhibitory activity and cytotoxicity of aqueous and ethanol extracts of *C. cornifolia* leaf and root part both *in vitro* and *in vivo*

- ***Courbonia glauca* (Klotzsch) Gilg & Bened** (Capparaceae)

**Fig. 1.4** Picture of *Courbonia glauca* plant, taken from the Malilangwe Wildlife Reserve, Mrewa, Zimbabwe

*Courbonia glauca* (Klotzsch) Gilg & Bened (Capparaceae) commonly called the Blue bush-cherry (English) Katunguru (Shona) Muswezu (Tonga: Zimbabwe) Soswe (Tonga: Zimbabwe) respectively (Hyde *et al.,* 2013). It is a small, glabrous, glaucous shrub up to 2 m tall but usually 1 m tall, with spreading branches from ground level or branching somewhat above the base. The fruit is said to be edible although some tribes in the Zambezi valley, Zimbabwe consider it necessary to cook it and throw away the water before eating. It is mostly distributed in Zimbabwe, Democratic Republic of Congo; Kenya; South Africa; Tanzania and Uganda (Wild, 1960; Hyde *et al.*, 2013)

According to the verbal communication survey we conducted in Mrewa and Mutoko districts in Zimbabwe in 2012. *C. glauca* was reported to be used to treat a number of disorder namely cancer, wound healing, bacterial infections, hypertension and diabetes. However, despite the extensive use of these plant parts in traditional medicine, scientific reports on the biological and pharmacological actions of this plant are limited. Based on our present knowledge few phytochemical investigations have been carried out on *C. glauca* (Maerua edulis) ethanol and aqueous extracts of the leaves and the roots, Luo *et al*. (2011) reported that n-hexane extract of *C. glauca* to possess six known fatty acid derivatives, including palmitic acid and its methyl ester, stearic acid, palmitoleic acid, oleic acid methyl ester and linoleic acid methyl. In view of its medicinal uses and the apparent lack of knowledge on its pharmacological properties, the

antioxidant, alpha glucosidase inhibitory activities and cytotoxicity profile of this plant was investigated in this study.

### 1.1.8 Statement of research problem.

Diabetes is an epidemic that is projected to continue on the rise, prominent to increased morbidity and mortality and greater expenditure of healthcare which will result in economies of developing countries being overburdened, much of the burden of diabetes mellitus for both patients and society comes from the long term complications associated with this disease. The worldwide survey reported that the diabetes mellitus (DM) is affecting nearly 10% of the world's population (Siddharth, 2001). The number of adults with diabetes worldwide is predicted to almost double over the next 25 years, from approximately 171 million in 2000 to 552 million by 2030 which is will be a quadruple from 2.8% to approximately 9.4% respectively(Chaturvedi, 2007;Gardner *et al.,* 2011).

In Africa, diabetes mellitus is no longer a rare disease and contemporary investigations of non-communicable diseases indicated prevalence from 1% to 20% of the population. The epidemiology has shifted from high mortality rate diseases such as AIDS, TB etc. to diabetes mellitus (IDF, 2011). In Zimbabwe and South Africa, the prevalence is between 4.5% and 6% and between 4% and 6% respectively (Deutschländer *et al*., 2009; Wild *et al*., 200; IDF, 2011). It is estimated that 80% of the affected are not aware of their condition and by the time they become aware the disease would have developed into complications which makes treatment even difficult (Wild *et al*., 2004).

Treatment of these related diseases and complications are far much beyond the reach of most developing countries budgets. The frequency of this disorder may increase tremendously on the population of developing countries; this is being perpetuated by to the lack of effective and affordable interventions of diabetes mellitus (Marx, 2002).

At present, out of all the currently available treatments for diabetes mellitus, none have proven to be highly effective, that is to completely cure the diseases and at the same time without causing severe side effects to the patient. The other set back with all available diabetes drug is that they

are expensive hence it makes them far beyond the reach of people in the developing countries, regardless of the fact that, that's where the disease is becoming much more prevalent.

Given the fact that the majority of the population in developing countries strongly rely on plants as their primary source of medicine and also in the light of the fact that more than 500 herbal medicines have been shown to possess antidiabetic properties both *in vitro* and *in vivo* (Honda *et al*., 2011; Ivorra *et al*., 1989). The scientific community has been therefore obliged to investigate intensively into these claims in trying to verify any possibility of the presence of medicinal value in most of the commonly used medicinal plants.

Additionally, medicinal plants have been widely used to treat a wide range of chronic metabolic disorders by traditional healers and herbalists especially in Africa (van Wyk, 2008). Compounds derived from natural products have immensely contributed to drug discovery process to a significant extend. This is shown by a big number of conventional drugs that are either derivative of natural products or are analogues of natural products (Ibrahim *et al.*, 2012). Thus, in recent years the screening of medicinal plants with potential antidiabetic properties has received considerable attention due to the increasing concern for safe and non-toxic alternative drugs (Aliyu *et al*., 2012).

Hence, the current study aims to expansively probe the anti-oxidative and antidiabetic activity of various solvent extracts of commonly used medicinal plants from the Mrewa district of Zimbabwe and with the aim of finding compounds that might be valuable in ameliorating diabetic related metabolic disorders as well as validating, in part, the traditional use of this plant in the treatment and management of diabetes.

### 1.1.9 Hypotheses:

Taking all the above facts into consideration, we hypothesize that:

Zimbabwean medicinal plants namely *Albizia antunesiana, Cissus cornifolia* and *Courbonia glauca,* which are commonly used for the treatment of diabetes and other ailments do possess antioxidants and antidiabetic activity, specific phytochemicals present in these plants are responsible for their pharmacological activities and they are relatively nontoxic.

- **Specific aims**
  - To evaluate the antioxidant, antidiabetic activity specifically (enzyme inhibiting activity) and cytotoxic activity of different plant extracts *in vitro.*

- **Specific Objectives**
  - To conduct a mini ethnobotanical survey (personal communication with traditional healers in Mrewa district in Zimbabwe) on the medicinal plants used to treat and manage diabetes.
  - To extract plant components using different solvent of increasing polarity (hexane (defatting agent), ethanol and water).
  - To investigate the antioxidant potential different of plant extracts *in vitro* using a series of antioxidant assays.
  - To evaluate the total phenolic content of aqueous and ethanolic plant extracts.
  - To use GC/MS analysis to identify different phytochemicals present in the plant extracts.
  - To assess the *in vitro* α-amylase and α-glucosidase inhibitory activities of the aqueous and ethanol extracts of the leaf and root part of the extracts of the above mentioned medicinal plants.
  - To determine the cytotoxicity effect of the aqueous and ethanol extracts of the leaf and root part of three (*Albizia antunesiana, Cissus cornifolia, Courbonia glauca*) plants in kidney cell lines.

### 1.1.10 Experimental design

In order to achieve the stated aim and objectives, this research was divided into relevant chapters below:

- **Chapter 2**

This chapter gives a detailed listing and description of all chemical reagents, equipment and *in vitro* assays used to investigate the above mentioned aim and objectives.

- **Chapter 3**

This chapter will provide detailed *in vitro* antioxidant activities and also *in vitro* α-amylase and α-glucosidase inhibitory effects and cytotoxicity activity of ethanol and aqueous extracts of *Albizia antunesiana* harms parts.

- **Chapter 4**

This chapter will provide detailed *in vitro* antioxidant activities and also *in vitro* α-amylase and α-glucosidase inhibitory effects and cytotoxicity activity of ethanol and aqueous extracts of *Cissus cornifolia* parts.

- **Chapter 5**

This chapter will provide detailed *in vitro* antioxidant activities and also *in vitro* α-amylase and α-glucosidase inhibitory effects and cytotoxicity activity of ethanol and aqueous extracts of *Courbonia glauca* parts.

- **Chapter 6**

This chapter gives a general discussion and also sum up all the previous chapters and put all the chapters in perception by linking and comparing the observed trends in each chapter and against other chapters. It highlights all significant findings from each and every chapter, drawbacks and also recommendations for further studies in the area of antioxidant, antidiabetic and cytotoxicity of medicinal plant extracts under study.

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# CHAPTER 2

## METHODOLOGY (MATERIALS AND METHODS)

## 2.1 Chemicals, reagents, apparatus and equipment

Garlic acid, acarbose, α-amylase, α-glucosidase, dinitrosalicylic acid, monosodium and disodium phosphate, ,ascorbic acid, 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), 2 deoxy-D-ribose, iron chloride, sodium carbonate, trichloroacetic acid, EDTA, 2-deoxy-D-ribose, pNPG(4-Nitrophenyl-D-glucopyranoside) and potassium ferricyanide were procured from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Griess reagent, sodium hydroxide, hydrogen peroxide, dimethyl sulfoxide (DMSO), sodium nitroprusside, thiobarbituric acid (TBA), Folin ciocalteau reagent and were purchased from Merck Chemical Company, Durban, South Africa. Starch was purchased from Ace associated chemicals enterprises, South Africa.

- **Glassware and plastic ware**

Beakers, conical and volumetric flasks, test tubes, schott bottles, measuring cylinders, cuvettes, pipette tips, latex examination gloves, Ziploc bags, Whatmann filter papers (No. 1, 2, 3)

- **Equipment**

Water bath, rotary evaporator (Buchi rotavapor II, Buchi Germany), electronic balance, UV-VIS Spectrophotometer (UV mini-1240 shimadzu) (Shimadzu Corporation, Kyoto, Japan), Pipettes(epperndoff), pH meter (schott), magnetic stirrer set, Freezer, Fume hood, Test tube rack, Timer, electric blender, Spatula, funnels, masking tape, paper foil, mortar and pestle, Freeze dryer, Agilent technologies 6890 Series GC coupled with an Agilent 5973 Mass Selective Detector and driven by Agilent chemstation software

## 2.2 Preliminary ethnobotanical survey

We conducted a mini preliminary ethnobotanical survey based on personal communications with local traditional healers and herbalist. Plants were selected based on the number of times there were mentioned for the folkloric treatment of diabetes mellitus. Based on the above mention criteria, the most cited five plants were selected these are: *Albizia antunesiana*, *Cissus cornifolia* and *Courbonia glauca*

- **Sample of ethnobotanical survey questionnaire**

AN ETHNOBOTANICAL SURVEY ON MEDICINAL PLANTS USED BY TRADITIONAL HEALERS IN MREWA AND MTOKO DISTRICT, MASHONALAND EAST PROVINCE OF ZIMBABWE

CONDUCTED

BY

CHIPITI TALENT (M.SC. STUDENT)

UNDER THE SUPERVISION OF MD. SHAHIDUL ISLAM

DESCIPLINE OF BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY, GENETICS AND MICROBIOLOGY, SCHOOL OF LIFE SCIENCES , COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

UNIVERSITY OF KWAZULU NATAL, DURBAN, SOUTH AFRICA

## A. Details of the traditional healer

I. First Name; ……, Surname……

II. Practice registration number……..

III. Physical address……
……
……

IV. Telephone number……..

V. Cell number…….

VI. Email address …….

## B. Declaration/consent statement

I, *(initial(s)*)……..., *(surname)* ……., have agreed to take part in this survey by providing relevant information and also help in the physical identification of the

plant(s) sample. All the information I will provide will be accurate and to the best of my knowledge.

**Signature**……...; **Date** ……..

**C. Details of the proposed plant.**

I. Local/traditional name…….
II. Botanical name…….
III. Physical description of the plant
……
……

**D. Physical sample and or photo, detailed description of the plant.**

| | |
|---|---|
| *Physical sample* | *Photo* |

I. In what places does this plant normally grows and in which season(s) ?.....
.....
..... .
II. What are the medicinal effect/value of the proposed plant ?……
……
……

III. Which part of the plant is used for medicine?.....
..... .

IV. How is the plant material prepared into medicine?.....
.....
.....
..... .

V. What is the effective dose administered to a patient at a given time?.....
..... .

VI. How is the prepared plant material (medicine), administered to a patient?.....
.....
..... .

VII. Are there any side effects known from the use of this medicine, Yes ☐

No ☐

VIII. If yes to the above question, what are the side effects?.....
.....
.....
..... .

IX. What indicates after administering the extract if its working?.....
.....
..... .

X. Besides the above mentioned plant is there any plant you are aware of that is used to treat and manage other diseases such as diabetes?……
…… .

XI. Is there any formulation you are aware of prepared from this plant and if any how effective is it?.....
.....

**THE END**

- **Plant collection, authentication and preparation**

Plants parts (leaves and roots) of the three plants namely *A. antunesiana, C. cornifolia,* and *C. glauca* were collected during the period of March 2012- February 2013 from Mrewa, Mashonaland East province, Zimbabwe. The plant samples were identified and authenticated by the herbarium unit of the Harare Botanical Garden and Herbarium, Harare, Zimbabwe and voucher specimens were deposited for *A. antunesiana, C. cornifolia,* and *C. glauca* with voucher numbers AA31509, CC082 and CG201 respectively.

These plant samples (leaves and roots) of the above mentioned plants were immediately washed with distilled water, cut into small pieces and shade-dried until constant weight was attained. The dried samples were ground to a fine powder using a blender, and stored individually in air-tight Ziploc bags and transported to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for further analysis. A bilateral contract has been signed between the University of KwaZulu-Natal, Durban, South Africa and Harare Botanical Garden and Herbarium, Harare, Zimbabwe before transporting these plant samples from Zimbabwe to South Africa

- **Preparation of plant extract**

The powdered plant part materials were subjected to sequential extraction in three solvent in order of increasing polarity (hexane, ethanol and water). Hexane was used specifically for defatting. Forty grams of the finely powdered plant part sample were separately defatted with hexane. The defatted materials were sequentially extracted with ethanol and water by soaking for 48 hours in 200 ml of the respective solvent. For ethanol extracts, after filtration through Whatmann filter paper (No. 1), the ethanol was evaporated under reduced pressure using a rotary evaporator (Buchi rotavapor II, Buchi Germany) at 40 $^{o}$C and the remaining ethanol was let to evaporate freely at room temperature. Aqueous extracts were dried using a freeze dryer. The

solvent extracts in each case was weighed, transferred to microtubes and stored in a refrigerator at 4-8 $^{o}$C until required.

### 2.3 Estimation of total phenolic content

The total polyphenol content of each extract was determined (as gallic acid equivalent) according to the method described by (McDonald *et al*., 2001) with slight modifications. Briefly, 200 µl of the extract (240 µg/ml) was incubated with 1 ml of ten-fold diluted Folin ciocalteau reagent and 800 µl of 0.7 M $Na_2CO_3$ for 30 min at room temperature. At the end of the incubation, absorbance values were determined at 765 nm using Shimadzu UV mini 1240 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate. The concentration of polyphenol was directly proportional to the absorbance of sample.

### 2.4 *IN VITRO* ANTIOXIDANT ASSAYS

#### 2.4.1 Ferric ($Fe^{3+}$) reducing antioxidant power assay (FRAP)

The FRAP method of (Oyaizu, 1986) with slight modifications was used to measure the reducing capacity of the extracts. To perform this assay, 1 ml of different extract concentrations (15–240 µg/ml) were incubated with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide at 50 $^{o}$C for 30 min. After 30 min incubation, the reaction mixture was acidified with 1 ml of 10 % trichloroacetic acid. Thereafter, 1 ml of the acidified sample of this solution was mixed with 1 ml of distilled water and 200 µl of $FeCl_3$ (0.1 %) in another test tube and the absorbance was measured at 700 nm in the above-mentioned Spectrophotometer. Increased absorbance of the reaction mixture indicates higher reduction capacity of the extracts which has been calculated according to the following formula:

$$\text{Ferric reducing antioxidant power (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ X } 100$$

#### 2.4.2 Free radical scavenging activity (DPPH Assay)

The free radical scavenging activity of the extracts was determined and compared to that of ascorbic and gallic acids by using a method described by Tuba and Gulcin, (2008) with slight

modifications. In order to perform this assay, a 0.3 mM solution of DPPH was prepared in methanol and 500 µl of this solution was added to 1 ml of the extract at different concentrations (15–240 µg/ml). These solutions were mixed and incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a blank lacking the scavenger.

The scavenging effects of the solvent extracts in the DPPH assay (including hydroxyl and nitric oxide radical assay as discussed below) were calculated according to the following equation:

$$\text{Scavenging activity } (\%) = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \text{ x } 100$$

Where: Ac is absorbance of control and As is absorbance of the sample or standard.

### 2.4.3 Hydroxyl radical scavenging (HRS) assay (Deoxyribose method)

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the solvent extracts for hydroxyl radicals generated by the ascorbate–EDTA–$H_2O_2$ system (Fenton reaction) as described by (Hinnerburg *et al*, 2006). The assay was performed by adding 200 µl of premixed 100 µM $FeCl_3$ ,100 µM EDTA (1:1 v/v) solution, 100 µl of 10 mM $H_2O_2$, 360 µl of 10 mM 2-deoxy-D-ribose, 1 ml of different extract concentrations (15–240 µg/ml), 400 µl of 50 mM sodium phosphate buffer (pH 7.4) and 100 µl of 1 mM ascorbic acid in sequence. The mixture was incubated at 50 $^{o}$C for 2 h. Thereafter, 1 ml of 2.8 % TCA and 1 ml of 1.0 % TBA (in 0.025 M NaOH) were added to each test tube. The samples were further incubated in a water bath at 50 $^{o}$C for 30 min to develop the pink chromogen colour. The extent of oxidation was estimated from the absorbance of the solution at 532 nm and the hydroxyl radical scavenging activity of the extract is reported as a percentage inhibition of deoxyribose degradation.

### 2.4.4 Nitric oxide (NO) radical scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions that can be estimated by using Griess reagent. Scavengers of NO compete with oxygen, leading to reduce the production of NO. The assay was carried out by incubating 500 µl of 10 mM sodium nitroprusside in phosphate buffer (pH 7.4) and 500 µl of different extract concentrations (15-240 µg/ml) at 37 $^{o}$C for 2 h.

The reaction mixture was then mixed with 500 µl of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. The percentage inhibition of nitric oxide generated was measured in comparison with the absorbance value of a control (sodium nitroprusside in phosphate buffer).

### 2.4.5 Gas chromatography-mass spectrometric (GC-MS) analysis

The aqueous and ethanol extracts of the leaf and root samples of the plant were subjected to GC-MS analysis. The analysis was conducted with an Agilent technologies 6890 Series GC coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent chemstation software. A HP-5MS capillary column was used (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The carrier gas was ultra-pure helium at a flow rate of 1.0 ml/min and a linear velocity of 37cm/sec. The injector temperature was set at 250 °C. The initial oven temperature was at 60 °C which was programmed to increase to 28.0 °C at the rate of 10 °C/min with a hold time of 4 min at each increment. Injections of 2 µl were made in splitless mode with a split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 4 min and scan range 50-700 amu. The compounds were identified by direct comparison of the retention times and mass spectral data and fragmentation pattern with those in the National Institute of Standards and Technology (NIST) library.

## 2.5 CARBOHYDRATE DIGESTING ENZYMES INHIBITION ASSAYS

### 2.5.1 α-amylase inhibitory activity of plant extracts

The α-amylase inhibitory activity of plant extracts was determined according to the method described by Shai *et al*. (2011) with slight modifications. A volume of 250 µL of each extract or acarbose at different concentrations (30-240 µg/mL) was incubated with 500 µL of porcine pancreatic amylase (2 U/mL) in 100 mM phosphate buffer (pH 6.8) at 37 $^{o}$C for 20 min. A 250 µL of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was then added to the reaction mixture and incubated at 37 $^{o}$C for 1 h. Then 1 mL of Dinitrosalicylic acid (DNS) colour reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at

540 nm and the inhibitory activity was expressed as percentage of a control sample without the inhibitors.

$$\text{Inhibition activity}(\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ X } 100$$

### 2.5.2 α-glucosidase inhibitory activity of plant extracts

The α-glucosidase inhibitory activity was determined according to the method described by Ademiluyi and Oboh (2013) with slight modifications. Briefly, 250 µL of the compound or acarbose at different concentrations (30-240 µg/mL) was incubated with 500 µL of 1.0 U/mL α-glucosidase solution in 100 mM phosphate buffer (pH 6.8) at 37 $^{o}$C for 15 min. Thereafter, 250 µL of pNPG solution (5 mM) in 100 mM phosphate buffer (pH 6.8) was added and the mixture was further incubated at 37 $^{o}$C for 20 min. The absorbance of the released p-nitrophenol was measured at 405 nm and the inhibitory activity was expressed as percentage of a control sample without the inhibitors.

$$\text{Inhibition activity}(\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ X } 100$$

## 2.6 *IN VITRO* CYTOXICITY STUDIES OF PLANT EXTRACTS ON CELL LINES

### 2.6.1 Cytotoxicity activity of plant extracts *in vitro* on HEK293 kidney cells using MTT assay

**Preparation of plant extracts**

Plant extracts were prepared (reconstituted in 10% DMSO), vortexed, filtered through Whatmann filter paper (No. 1)and left for 15 min, before further dilution with the respective growth medium) and tested on kidney cells.

- **Cell line maintenance**

Routine cell culture maintenance of the human embryonic kidney cells (HEK293) was done by, incubating the cells at 37◦C in a humidified atmosphere supplemented with 5% $CO_2$. Cells were replenished with fresh growth medium every 2–3 days, consisting of media (Minimum Essential Medium + Glutmax + antibiotics+10% fetal bovine serum).

- **MTT Cell proliferation assay**

Aqueous and ethanol extracts were tested for *in vitro* cytotoxicity, using HEK293 kidney cells by 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983; Page-McCaw *et al*., 2007). Confluent monolayer culture suspensions of the cells were trypsinised and plated into 96 well plates at a seeding density of $2.5 \times 10^3$ cells per well and incubated for 24 h at 37 $^oC$ in a 5% $CO_2$ incubator in a culture medium containing 10% FBS. Following 24-h incubation and attachment, the cell culture medium was replaced with fresh medium. Thereafter, varied concentrations of plant extracts (50–200 µg/mL) were added in triplicate to the cells and the plate incubated for 48 h as previously. Two controls, one containing only cells and one containing DMSO were also set up. After 48 hrs, all culture media was removed from the plates, the cells were washed with PBS, and 100µL of the cell media and 100µL of MTT solution (5 mg/mL in phosphate buffered saline) was added to each well. The plates were then incubated for 4 h at 37 $^oC$. Thereafter, 100µL DMSO solution was added to each well to stop the reaction and dissolve the insoluble formazan crystals. Absorbance was measured at 570 nm using a Mindray MR-96A microplate reader. The assessment of cytotoxicity was based on a comparison with untreated cells and expressed as $IC_{50}$ (the concentration of the sample required to inhibit 50% of cell proliferation), calculated from the dose–response curve (curve fit–nonlinear regression, four parameter). The values are presented as means of triplicate analyses.

### 2.7 Statistical Analysis

All data are presented as the mean ± SD of triplicates determination. Data were analyzed by SPSS statistical software (version 19, Windows IBM Inc., USA) using Tukey's multiple range post-hoc tests. Values were considered significantly different at $p < 0.05$.

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# CHAPTER 3

## *In vitro* antioxidant, anti-hypergycemic and cytotoxicity activity of *Albizia antunesiana* extracts.

# 3.1 I*n vitro* antioxidant activities of leaf and root extracts of *Albizia antunesiana* harms

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**Running title:**

Anti-oxidative effects of *Albizia antunesiana* harms.

### 3.1.1 Preface

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### 3.1.2 Abstract

The anti-oxidative activities of the ethanol and aqueous extracts of the leaf and root samples of *Albizia antunesiana* were determined across a series of four *in vitro* models. The results showed that all the extracts had reducing power ($Fe^{3+}$- $Fe^{2+}$), DPPH, hydroxyl and nitric oxide radical scavenging abilities. The ethanol root extract had more potent antioxidant power in all the experimental models and to possess a higher total phenol content of 216.6 ± 6.7 mg/g. The GC-

MS analysis of the aqueous and ethanol extracts of the roots and leaves indicated that several aromatic phenolic compounds, a coumarin and some common triterpenoids were present in these extracts. Data from this study suggest that the leaves and roots of *A.antunesiana* possessed anti-oxidative activities that varied across the solvents.

**Key words:** Anti-oxidative, *in vitro, Albizia antunesiana*, GC-MS

### 3.1.3 Introduction

Oxidative stress is a biochemical anomaly caused by disparity between the absolute overload of oxidants from the normal physiological and biochemical pathways and a successive depletion of antioxidant components in the body. It is involved in the pathogenesis of a vast number of chronic metabolic disorders which include atherosclerosis, ischaemia/reperfusion injury, chronic inflammatory diseases, renal failure and diabetes mellitus (Calabrese and Cornelius, 2012). Hence, the devastating effects of oxidative stress in a number of chronic metabolic disorders have compelled the scientific community to search for antioxidative compounds which can inhibit the oxidation of oxidizable molecules in a chain reaction that could consequently be vital in the therapy and prevention of these disorders (Wright *et al*., 2006). Unfortunately, although synthetic antioxidative agents, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tertiobutylhydroxytoluene exhibit potent free radical scavenging effects, they induce liver damage and carcinogenesis in laboratory animals (Saito *et al*., 2003; Subhasree *et al*., 2009; Djeridane *et al*., 2010). Therefore, there is a need for the development and utilization of more effective antioxidants of natural origin (Djeridane *et al*., 2010).

Medicinal plants have been widely used for the treatment of a range of chronic metabolic disorders by traditional healers and herbalists especially in developing countries in Africa where more than 5400 medicinal plants have been reported to have over 16000 medicinal uses (van Wyk, 2008; Ibrahim *et al*., 2012). Compounds obtained from natural products have influenced the field of drug discovery to a greater magnitude. This is evident in a number of clinically active drugs that are either natural products or analogues of natural product (Koehn and Carter, 2005; Ibrahim *et al*., 2012). Thus, in recent years the screening of medicinal plants with potential

antioxidant properties has received considerable attention due to the increasing concern for safe and non-toxic alternative antioxidants (Aliyu *et al*., 2012).

*A.antunesiana* Harms (Fabaceae), commonly called purple-leaved albizia, is indigenous to Zimbabwe. Locally, it is called "Muriranyenze" and "Umnonjwana" by the Shona and Ndebele-speaking Zimbabweans, respectively. The leaves and roots of this plant are used by traditional healers to cure several metabolic and non-metabolic disorders such as sore eyes, cuts, ulcers, sore throat, tonsillitis, tuberculosis, gonorrhea, diabetes and cardiac problems (Ndemera *et al*., 1985). However, despite the extensive use of these plant parts in traditional medicine, scientific reports on the biological and pharmacological actions of this plant are limited.

**Fig. 3.1** Picture of *A.antunesiana* harms. Picture taken from Chivake river Mrewa, Zimbabwe.

Hence, the current study aims to expansively probe the anti-oxidative effects of various solvent extracts of the leaves and root with the aim of finding compounds that might be valuable in ameliorating oxidative stress mediated chronic metabolic disorders as well as validating, in part, the traditional use of this plant in the treatment and management of a vast array of chronic metabolic disorders.

### 3.1.4 Materials and methods

Please refer to the section **2.1 – 2.4** and **2.7** in pages (**43 – 54**) for detailed materials and methods.

### 3.1.5 Results

**Table 3.1:** Percentage recovery (g/g) and total phenolics content of various solvent extracts of *A. antunesiana* parts

| Samples | % recovery (g/g) | Total polyphenol (mg/g GAE) |
|---|---|---|
| **Leaves** | | |
| EtOH | 4.36 | $134.3 \pm 13.5^{b}$ |
| Aqueous | 4.79 | $121.5 \pm 13.5^{b}$ |
| **Root** | | |
| EtOH | 1.59 | $216.6 \pm 6.70^{c}$ |
| Aqueous | 7.10 | $109.1 \pm 6.20^{a}$ |

Data are presented as mean ± SD values of triplicate determinations. $^{a-c}$Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The percentage recovery (g/g) obtained from various extracts (**Table 3.1**) collected from the plant indicated that the highest recovery was obtained in the aqueous extracts. All extracts were found to possess a high total phenolic content. However, the highest total phenolic content was recorded in the ethanol extract of the roots (**Table 3.1**).

**Table 3.2** shows the total reducing power of the ethanol and aqueous extracts of *A. antunesiana* parts. All the extracts showed an ability to donate electrons to convert $Fe^{3+}$-$Fe^{2+}$ as indicated by the concentration dependent increase in the percentage reducing power. The ethanol extract of the root had a significantly ($P<0.05$) higher total reducing power than other extract.

**Table 3.2**: Percentage total reducing power (GAE) of solvent extracts from various parts of *A. antunesiana*

| Extracts | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| | 15 | 30 | 60 | 120 | 240 |
| **Leaves** | | | | | |
| EtOH | 16.38 ± 1.30[a] | 21.19 ± 2.45[a] | 26.37 ± 1.33[b] | 27.93 ±1.14[a] | 49.85 ± 9.07[a] |
| Aqueous | 8.91 ± 3.39[a] | 12.62 ± 4.54[a] | 14.50 ± 0.88[a] | 18.12 ± 5.69[a] | 39.13 ± 1.00[b] |
| **Root** | | | | | |
| EtOH | 36.08 ± 1.12[b] | 49.47 ± 2.66[b] | 50.64 ± 0.45[c] | 54.89 ± 2.39[b] | 85.76 ± 1.05[b] |
| Aqueous | 11.46 ± 2.02[a] | 21.04 ± 0.20[a] | 25.18 ± 0.63[b] | 32.71 ± 1.80[b] | 49.21 ± 1.10[b] |
| Standard | | | | | |
| Ascorbic acid | 76.83 ± 4.92[c] | 80.24 ± 3.00[c] | 90.26 ± 5.51[d] | 91.08 ± 5.43[b] | 93.60 ± 6.21[b] |

Data are presented as mean ± SD values of triplicate determinations. [a-d] Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

**Fig. 3.2** shows the DPPH radical scavenging activities of the leaf (A) and root (B) extracts of *A. antunesiana* parts. All the extracts showed an ability to quench DPPH free radicals as indicated by the concentration dependent increase in the percentage inhibition. The root extracts had a consistently higher DPPH radical scavenging activity, which is concentration dependent (**Fig. 3.2**).

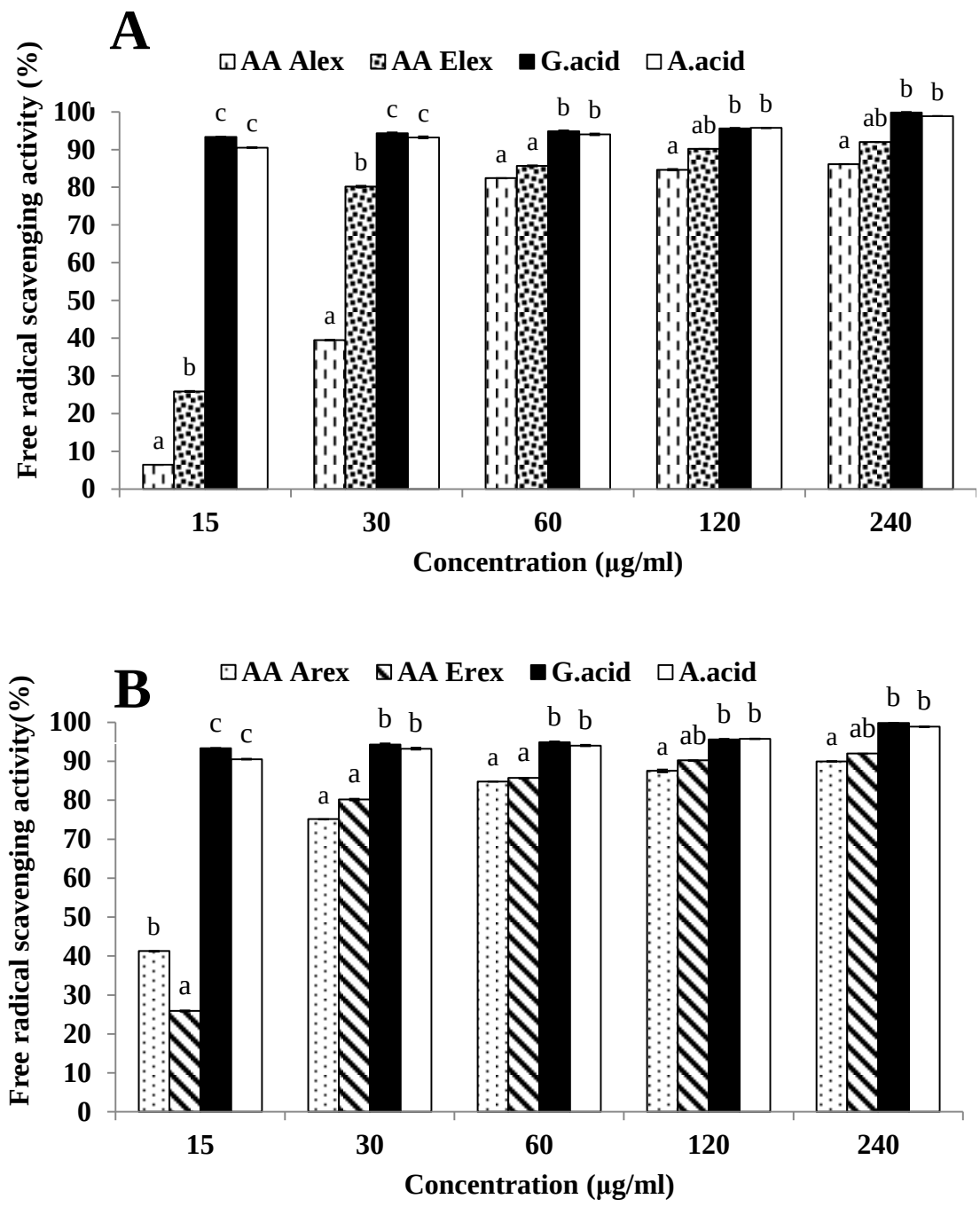


**Fig. 3.2**: DPPH radical scavenging activity of ethanol and aqueous extracts of the leaves (**A**) and root (**B**) of *A.antunesiana*.

Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD of triplicate determinations. [a-c]Values with different letters over the bars for a given concentration of each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

**Table 3.3**: Percentage hydroxyl radical scavenging activity of extracts from the root and leaves of *A. antunesiana*

| | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| | 15 | 30 | 60 | 120 | 240 |
| *Extracts* | | | | | |
| **Leaves** | | | | | |
| Ethanol | 34.96 ± 0.04$^{d}$ | 51.63 ± 0.05$^{d}$ | 56.00 ± 0.51$^{d}$ | 65.67 ±1.40$^{d}$ | 83.00 ± 2.00$^{c}$ |
| Aqueous | 4.76 ± 1.83$^{a}$ | 8.90 ± 4.22$^{a}$ | 13.09 ± 4.11$^{a}$ | 18.42 ± 3.10$^{a}$ | 26.87 ± 8.10$^{a}$ |
| **Root** | | | | | |
| Ethanol | 22.50 ± 2.33$^{c}$ | 29.00 ± 1.94$^{c}$ | 40.04 ± 0.53$^{d}$ | 56.90 ± 0.40$^{d}$ | 63.00 ± 0.50$^{d}$ |
| Aqueous | 0.80 ± 0.05$^{a}$ | 2.50 ± 0.92$^{a}$ | 5.50 ± 3.40$^{a}$ | 13.30 ± 0.73$^{a}$ | 27.60 ± 0.71$^{a}$ |
| **Standards** | | | | | |
| Ascorbic acid | 14.50 ± 2.20$^{b}$ | 17.35 ± 3.10$^{b}$ | 21.85 ± 0.10$^{b}$ | 26.80 ± 0.70$^{b}$ | 30.30 ± 1.20$^{a}$ |
| Gallic acid | 32.00 ± 0.60$^{d}$ | 35.57 ± 2.30$^{d}$ | 36.75 ± 1.80$^{c}$ | 38.90 ±1.10$^{c}$ | 40.20 ± 0.20$^{c}$ |

Data are presented as mean ± SD values of triplicate determinations. $^{a-d}$Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

The results of the HRS assay indicated that all (ethanol and aqueous) extracts could scavenge hydroxyl radicals generated by Fenton's reaction with the ethanol extracts displaying significantly higher scavenging abilities compared to the aqueous extracts and the standards used (**Table 3.3**). It is also notable that the ethanol extract of the leaf consistently demonstrated a significantly higher ($P<0.05$) HRS activity than all other extract.

**Table 3.4**: Percentage NO scavenging activities of extracts from various parts *A. antunesiana*

| | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| ***Extracts*** | **15** | **30** | **60** | **120** | **240** |
| **Leaves** | | | | | |
| Ethanol | 49.27 ± 0.80$^{b}$ | 49.36 ± 1.50$^{b}$ | 55.47 ± 0.40$^{c}$ | 55.47 ± 0.30$^{b}$ | 56.99 ± 0.21$^{b}$ |
| Aqueous | 16.80 ± 1.20$^{a}$ | 28.00 ± 0.90$^{a}$ | 33.6 ± 0.70$^{a}$ | 35.11 ± 0.70$^{a}$ | 43.51 ± 2.20$^{a}$ |
| **Root** | | | | | |
| Ethanol | 28.24 ± 1.60$^{b}$ | 37.15 ± 1.30$^{b}$ | 43.66 ± 0.80$^{c}$ | 46.54 ± 0.70$^{c}$ | 58.57 ± 1.10$^{c}$ |
| Aqueous | 25.44 ± 6.30$^{b}$ | 36.64 ± 2.70$^{b}$ | 39.44 ± 0.70$^{c}$ | 42.23 ± 0.90$^{c}$ | 55.21 ± 0.20$^{c}$ |
| **Standards** | | | | | |
| Ascorbic acid | 51.44 ± 0.05$^{b}$ | 56.94 ± 0.90$^{b}$ | 58.37 ± 1.40$^{c}$ | 62.44 ± 0.70$^{b}$ | 77.22 ± 1.20$^{c}$ |
| Gallic acid | 43.06 ± 9.20$^{b}$ | 47.36 ± 0.70$^{b}$ | 49.76 ± 0.70$^{b}$ | 59.33 ± 3.20$^{b}$ | 64.59 ± 6.20$^{b}$ |

Data are presented as mean ± SD values of triplicate determinations. $^{a\text{-}c}$Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

**Table 3.4** presents the NO inhibition activities of ethanol and aqueous extracts of *A. antunesiana* leaves and roots. All the extracts were found to exhibit NO inhibition activity but the ethanol extract of the leaves demonstrated a higher NO inhibition effect than other extracts. Furthermore, the ethanol extracts of the leaves and roots possessed lower $IC_{50}$ values of 20.42 and 15.95 µg/mL respectively, than the corresponding aqueous extracts in these plant parts (**Table 3.5**).

**Table 3.5:** $IC_{50}$ values of various solvent extracts of *A. antunesiana* parts in different anti-oxidative models

| | IC 50 values in µg/ml | | |
|---|---|---|---|
| Samples | DPPH | HRS | NO |
| **Leaves** | | | |
| Ethanol | 42.65 ± 2.45 [b] | 34.67 ± 8.90 [b] | 20.42 ± 3.79 [a] |
| Aqueous | 45.70 ± 3.22 [b] | 6.02 ± 178.98 [a]* | 501.18 ± 23.47 [d] |
| **Root** | | | |
| Ethanol | 7.08 ± 0.57 [a] | 97.72 ± 11.22 [d] | 15.95 ± 4.76 [a] |
| Aqueous | 12.58 ± 1.25 [a] | 4.36 ± 304.22 [a]* | 223.87 ±16.78 [c] |
| **Standards** | | | |
| Ascorbic acid | 5.01 ± 0.76 [a] | 64.65 ± 7.78 [c] | 15.13 ± 3.22 [a] |
| Gallic acid | 5.62 ± 2.34[a] | 6.30 ± 205.67 [e]* | 41.68 ± 2.90 [b] |

The * represent the units of these values which are in mg/ml. Data are presented as mean ± SD values of triplicate determinations. [a-e]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range post hoc test, $P<0.05$)

Identified compounds of EtOH and aqueous extracts of different parts of *A.antunesiana* by GC-MS, from all extracts of ethanol and aqueous aromatic compounds which were identified are presented in **Table 3.6**. Benzofuran was present in both extracts of the leaf and isomers catechol and resorcinol were all present in root extracts of both, much aromatic compounds like pyrogallol, alpha and beta amyrin were present in the ethanolic root extract.

Phenolic compounds identified in the root and leaf extracts of *A.antunesiana*, the identified represented in the **Fig. 3.3** which all do possess a free hydroxyl group attached to it which makes them ideal antioxidant compounds.

**Fig. 3.4** is a schematic illustration of a proposed mechanism by which the identified phenolic compounds act to neutralise free radicals generate during bodys normal physiological processes like aerobic respiration

**Table 3.6**: Identified compounds of EtOH and aqueous extracts of different parts of *A.antunesiana* by GC-MS

| **Compounds** | **Retention time (min)** | **Molecular mass(g/mol)** |
|---|---|---|
| ***Ethanol extract*** | | |
| **Leaves** | | |
| Benzofuran | 7.69 | 118.13 |
| **Roots** | | |
| Catechol | 7.66 | 110.10 |
| Resorcinol | 8.69 | 110.10 |
| Pyrogallol | 9.91 | 126.11 |
| Alpha-amyrin | 22.63 | 426.73 |
| beta amyrin | 22.63 | 426.73 |
| ***Aqueous extract*** | | |
| **Leaves** | | |
| Benzofuran | 7.69 | 118.13 |
| Methyl mannitol | 10.55 | 126.11 |
| pyrogallol | 18.03 | 262.30 |
| **Root** | | |
| Catechol | 7.75 | 110.10 |
| Resorcinol | 8.81 | 110.10 |
| Pyrogallol | 10.12 | 126.11 |

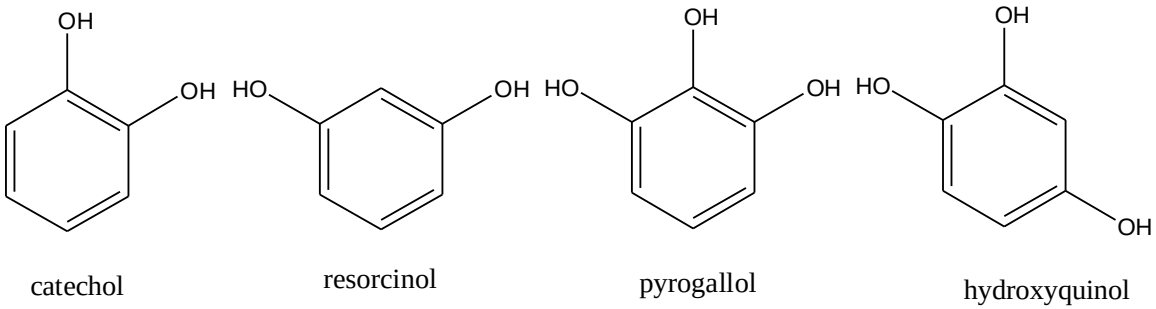


**Fig. 3.3.** Phenolic compounds identified in the root and leaf extracts of *A.antunesiana*

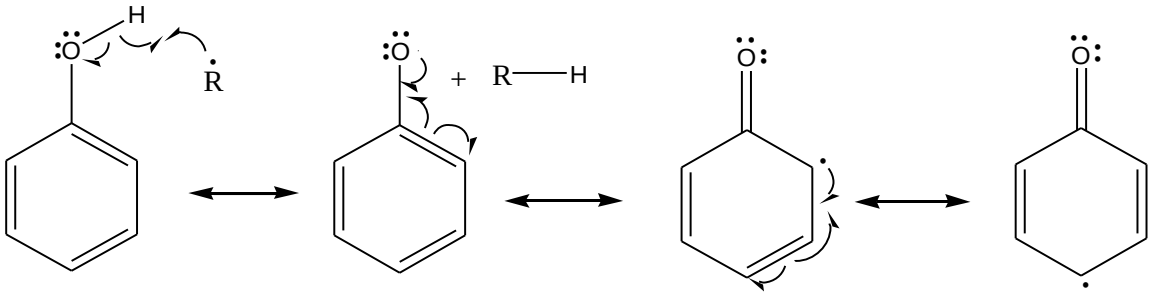


**Fig. 3.4.** Hydrogen radical donation to a reactive radical species and stabilization of the resultant radical

### 3.1.6 Discussion

*Albizia antunesiana* is used by traditional healers and herbalist to treat and manage non metabolic chronic disorders in Mrewa district Zimbabwe (personal communication with traditional healers). The genus *Albizia* comprises over 150 species across the world with many species being used for medicinal purposes but only a few have been investigated for biological activities (Singh *et al*., 2004). In this study, we determined the complete anti-oxidative profile of the ethanol and aqueous extracts from the leaves and roots as a lead in the search for extracts with potential compounds that could be useful in alleviating oxidative stress-associated complications. Our findings also indicate that some *A. antunesiana* extracts possess exceptionally high anti-oxidative activities, as proven by the *in vitro* experimental models.

In the present study, the set of *in vitro* assays were employed to cover all possible known mechanisms by which different antioxidants operate to inhibit oxidative chain reactions since there is no single model known to mimic all oxidative pathways. The ferric reducing antioxidant

assay (FRAP) which measures the total reducing ability was measured as ferric ($Fe^{3+)}$ to ferrous ($Fe^{2+}$) conversion in the presence of the extracts using the protocol of Oyaizu .(1986). This assay is relevant due to its ability to assess the extract potential to facilitate the conversion of ($Fe^{3+)}$ to ($Fe^{2+}$). The extent to which it promotes this reduction process will indicate how effective the extract is as an antioxidant compound. The significantly ($P<0.05$) higher antioxidant ability indicated by the ethanol extract of the root and leaf samples indicate that the phytochemical components with high redox potential in these plant parts are more soluble in ethanol than water.

DPPH is a stable molecule consisting of a nitrogen-centered free radical with characteristic color changes from violet to yellow upon reduction by either the process of hydrogen- or electron-donation. The degree of the reduction is reflective of the radical scavenging (antioxidant) power of the compound (Hinnerburg *et al*., 2006). The results of the DPPH assay of the aqueous and ethanol extracts of the leaves and roots revealed that virtually all extracts possessed stronger free radical scavenging potential compared to gallic and ascorbic acids.

However, the $IC_{50}$ values indicated the best free radical scavenger to be ethanol root extracts because of the low value of 7.08 µg/mL which is in the same range with that of ascorbic and gallic acids. This could further indicate that these extracts contain powerful free radical scavenging phytochemicals that might have the potential to inhibit free radical accumulation which usually build up in chronic metabolic disorders and therefore could be useful therapeutic agents for treating free radical linked pathological conditions.

The hydroxyl radicals are highly reactive free radicals formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living systems (Ibrahim *et al*., 2012). The hydroxyl radical can target and damage virtually all types of major macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids (Pastor *et al*., 2000). Henceforth, the identification of plant extracts which exhibit high hydroxyl radical quenching potential will be useful for the prevention of the hydroxyl radical-mediated destruction of biological molecules against oxidative stress related diseases.

The hydroxyl radical scavenging activity was measured as the percentage inhibition power of the extract towards hydroxyl radicals generated from the Fenton's reaction mixture by studying the

competition between deoxyribose and the extract for hydroxyl radicals generated from $Fe^{3+}$/ascorbate/EDTA/$H_2O_2$ systems (Ibrahim *et al*., 2012). Our results indicated that all extracts had a potential to scavenge hydroxyl radicals. However, the ethanol extracts have proven, at least under *in vitro* conditions, to contain better antioxidants for use against diseases where the hydroxyl radical upsurge is an important mechanism for pathogenesis.

Nitric oxide, a chemical intermediary generated by endothelial cells, macrophages, and neurons is involved in a variety of physiological processes (Lata and Ahuja, 2003; Ibrahim *et al*., 2012). Excess concentration of nitric oxide is associated with a number of diseases (Ross *et al*., 1993). Oxygen reacts with excess nitric oxide to form nitrite and peroxynitrite ions, which act as free radicals. Our present study indicated that all the extracts have a competing power with oxygen to react with nitric oxide and thus inhibition of anions to some extent, which varies across different extracts. All the extracts did not show much promising nitric oxide scavenging ability as compared to other antioxidant activities. This difference can be accounted for by the way in which the nitric oxide radicals are generated and the different physical and chemical properties of the antioxidants contained in the extracts (Schwarz *et al*., 2001)). The difference in antioxidant activity using different stable radicals $Fe^{2+}$/$Fe^{3+}$ might be influenced by different redox potentials and steric properties (Gardner *et al*., 1998).

GCMS analysis of the aqueous and ethanol extracts of the leaves and roots were carried out in order to identify the presence of aromatic and phenolic compounds capable of having antioxidant activity. Two isomers of benzenediol (catechol and resorcinol) and two isomers of benzenetriol (pyrogallol and hydroxyquinol) were identified in the extracts by comparing their MS spectra to those of standard spectra from the NIST library. Catechol, pyrogallol and resorcinol were found in the ethanol and aqueous extracts of the roots while pyrogallol and hydroxyquinol were found in the aqueous extract of the leaves. In addition, coumarin was found in the ethanol and aqueous extracts of the leaves and α- and β-amyrin, two well know triterpenoid natural products were detected in the ethanol extracts of the roots. Phenolic compounds such as those described here (**Fig. 3.3**) are capable of donating a hydrogen radical or an electron to quench radicals as the resultant aromatic radicals are able to be stabilized by delocalization of the resultant radical on the benzene ring (**Fig. 3.4**).

Overall, we concluded that the observed antioxidant activity with the EtOH root extract might be linked to the high phenolic content which has been shown by the higher activity of this extract. The ethanol root extract has indicated remarkably higher activities across *in vitro* anti-oxidant models used, which could prompt more studies to be done in order to identify and purify the active compounds and their underlying mechanisms. Furthermore, the results displayed by the ethanol root extract are interesting enough to be used in *in vivo* models. Interestingly, findings from this study also support the use of the root by traditional healers in the Mrewa district as medicine.

### 3.1.7 Post-script analysis

*A.antunesiana* extracts were further subjected to *in vitro* α-amylase, α-glucosidase inhibitory and cytotoxicity assays to further investigate the possible mechanism of antidiabetic action and the results were presented in the following section.

# *3.2 In vitro* α-amylase and α-glucosidase inhibitory effects and cytotoxicity activity of ethanol and aqueous extracts of *Albizia antunesiana* harms parts

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Running tittle:

Antidiabetic and cytotoxicity activity of *Albizia antunesiana* harms parts.

### 3.2.1 Preface:

This section of the chapter is still under construction into a manuscript but not yet submitted to the journal for publication.

### 3.2.2 Abstract

Diabetes mellitus is a chronic disease and its incidence is tremendously increasing globally. One therapeutical approach is by decreasing postprandial hyperglycemia by retarding glucose absorption through inhibiting carbohydrates digesting enzymes. *Albizia antunesiana* aqueous and ethanol extracts were investigated for α-amylase and α-glucosidase inhibitory activity *in vitro.* The results show that ethanol root extract of *A.antunesiana* had mild α-amylase inhibition

activity and strong α-glucosidase inhibition power also supported by $IC_{50}$ values (30.68 and 4.35 µg/ml). The aqueous root extract showed mild inhibition of α-glucosidase but non α-amylase inhibition power. The ethanolic (leaf and root) extracts shows to be relatively non-toxic at tested concentrations on the HEK 293 cell lines as confirmed by the MTT assay. However the aqueous extracts (leaf and root) were cytotoxic at concentrations above 50 µg/ml. Data from this study suggest that the ethanolic extracts of roots of *Albizia antunesiana* possessed α-amylase, α-glucosidase inhibitory activity *in vitro* and did not cause significant cell death on the tested concentrations.

### 3.2.3 Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is increasing at an alarming rate worldwide such that it is currently being estimated to be responsible for 90-95% of all diabetes cases (Wild *et al.*, 2004). This is as a result of life-style and socioeconomic changes mainly characterized by a decrease of physical activity and an increase in high fat intake among other factors (IDF, 2011).

Hyperglycemia is a hall mark of T2DM and plays vital roles in most of the pathogenic features of the disease. Hyperglycemia, a condition which prevails when there is decreased insulin sensitivity or decreased insulin secretion from pancreatic β-cells, can further inhibit insulin secretion from pancreas and reduce insulin-mediated glucose uptake in peripheral tissues (Wolffenbuttel and Haeften, 1995; Shaiq *et al.*, 2002). All diabetic complications (nephropathy, neuropathy, microangiopathy, macroangiopathy, retinopathy and cataract) are strongly linked to hyperglycemia. This calls for improved treatment of hyperglycemia, T2DM-related risk factors and the long-term degenerative disorders so as to dramatically lower the risk of both micro- and macrovascular complications.

An important strategy to control hyperglycemia is through the inhibition of key carbohydrates digesting enzymes such as α-amylase and α-glucosidase which is also vital in preventing diabetic complications. The inhibitors of these enzymes (α-amylase and α-glucosidase) delays the digestion of carbohydrates, thereby prolonging the overall carbohydrate digestion time and reduces the rate at which glucose is absorbed from the gut, thereby lowering the postprandial rise

in blood glucose. Thus, inhibition of α-amylase and α-glucosidase is a key in the management and treatment of T2DM (Shaiq *et al.*, 2002).

Currently, there are several conventional prescribed α-glucosidase inhibitor drugs which include acarbose, voglibose and miglitol; however, there have been shown to have some undesirable adverse effects such as flatulence, diarrhea and abdominal pain which were hostile to patients (Shaiq *et al.*, 2002). This indicates the urgent need for the development of newer alternatives. A better clinical outcome could be derived from specific α-amylase and α-glucosidase inhibitors with strong inhibitory activity against the α-amylase and α-glucosidase and not cytotoxic to target cells, but still achieved the desired result of delaying postprandial hyperglycemia. An ideal compound or drug will be the one that will mildly inhibit α-amylase and strongly inhibiting α-glucosidase (Krentz and Baile, 2005).

The use of medicinal plants have been adopted since ancient times to treat a lot of ailments, some of the plants and plant derived products have shown impressing potentials such that they influence the discovery of some current conventional medicines. Some medicinal plants have been shown to possess other mechanisms of action, which includes α-amylase and α-glucosidase inhibition activities (Ortiz-Andrade *et al*., 2007; Shirwaikar *et al*., 2005). Unfortunately, some reports indicated the presence of some potential toxic and carcinogenic (Fennell *et al*., 2004) agents in some of these plants, making them unsuitable for therapeutic applications. It is therefore of the utmost important to intensively investigate the potential cytotoxic activity in order to validate safety for the continued use of medicinal plants. It has been documented that some plants extracts do have bioactivity however it is cancelled by their cytotoxicity; hence such scenarios need to be evaluated so as to assess the overall efficacy of the plant extract (Fennell *et al*., 2004).

*Albizia antunesiana* Harms (Fabaceae), commonly called purple-leaved albizia, is indigenous to Zimbabwe. Locally, it is called "Muriranyenze" and "Umnonjwana" by the Shona and Ndebele-speaking Zimbabweans, respectively. The leaves and roots of this plant are used by traditional healers to cure several metabolic and non-metabolic disorders such as sore eyes, cuts, ulcers, sore throat, tonsillitis, tuberculosis, gonorrhea, diabetes and cardiac problems (Ndemera *et al*., 1985). However, despite the extensive use of these plant parts in traditional medicine, scientific reports on the biological and pharmacological actions of this plant are limited. In a recent study,

we reported the anti-oxidative activities of the aqueous and ethanol extracts of the leaves and root of the plant as well as their possible bioactive compounds (Chipiti *et al*., 2013). Hence, the present study was therefore undertaken to intensively probe the *in vitro* α-amylase and α-glucosidase inhibitory effects and cytotoxicity activities of the ethanol and aqueous extracts of this plant as potential sources of nontoxic therapeutically anti-diabetic agents which can be of use in achieving normoglycaemia.

### 3.2.4 Materials and methods

Please refer to the section **2.1 - 2.2** and **2.5 - 2.7** in pages (4**3 - 54**) for detailed materials and methods.

### 3.2.5 Results

Fig 3.5 shows inhibitory activity of *A.antunesiana* aqueous and ethanol extracts on α-amylase (Fig 3.5A) and α-glucosidase (Fig 3.5B). The result indicates that *A.antunesiana* ethanol root extract is a better α-amylase inhibitor than the standard acarbose. However, aqueous root and both leaf extracts do possess inhibition potential which is significantly lower ($P<0.05$) than that of the standard. Fig 3.5B also indicated the ability of root extracts to have better inhibitory activity on α-glucosidase than acarbose, however the inhibitory activity was much pronounced in the ethanol root extract because it had significantly higher ($P<0.05$) α-glucosidase inhibition at all concentrations tested.

The $IC_{50}$ values for inhibiting α-glucosidase (4.35 ± 0.56 μg/mL) and α-amylase (30.68 ± 1.09 μg/mL**)** of the ethanol root extract of indicates that the extract is a moderate inhibitor of α-amylase and a potent inhibitor of α-glucosidase (Table 3.7).

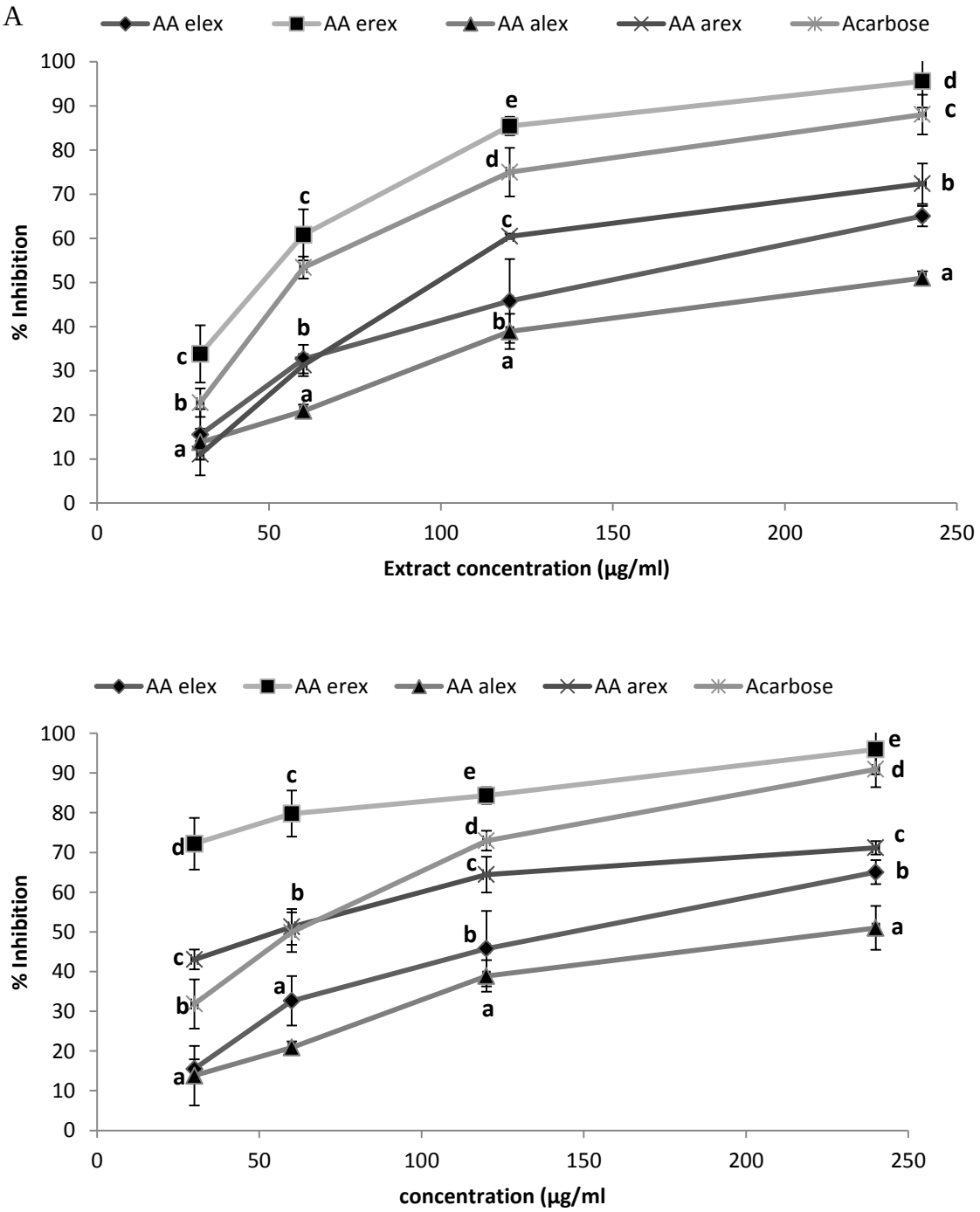


**Fig. 3.5: A** and **B** = α-amylase and α-glucosidase respectively shows inhibitory activity of *A.antunesiana* aqueous and ethanol extracts. Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD values of triplicate determinations. [a-e]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range post hoc test, $P<0.05$).

**Table 3.7**: $IC_{50}$ values for α-amylase and α-glucosidase inhibition activity of *A.antunesiana* extracts

| | IC50 values (µg/mL) | |
|---|---|---|
| **Name of extract** | **α-Amylase** | **α-Glucosidase** |
| **Aa elex** | 85.78 ± 3.12 [c] | 131.67 ± 14.22 [d] |
| **Aa alex** | 345.67 ± 12.89 [e] | 232.48 ± 21.03 [e] |
| **Aa erex** | 30.68 ± 1.09 [a] | 4.35 ± 0.56 [a] |
| **Aa arex** | 102.87 ± 9.80 [d] | 49.86 ± 2.09 [b] |
| **Acarbose** | 52.11 ± 0.56[b] | 57.18 ± 3.54[c] |

Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD values of triplicate determinations. [a-e]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

Fig. 3.6 displays the cytotoxicity activity of the *A.antunesiana* extracts on HEK 293 kidney cell lines as confirmed by MTT assay. As indicated in Fig 3.6 *A.antunesiana* ethanol root extracts did not cause any decrease cell viability to significance extent however the aqueous( leaf and root) extracts displayed a significant decrease in cell viability across all tested concentrations (50 – 200 µg/ml), the cell viability was decreasing as the concentration of the extract was increasing. This is also consistent with the high $IC_{50}$ (Table 3.8) value obtained for *A.antunesiana* ethanol root extract (6.14 ± 325.01 mg/ml).The aqueous root extract was the most toxic indicated to have the lowest $IC_{50}$ (Table 3.8) value of 206.71 ± 13.90 mg/ml.

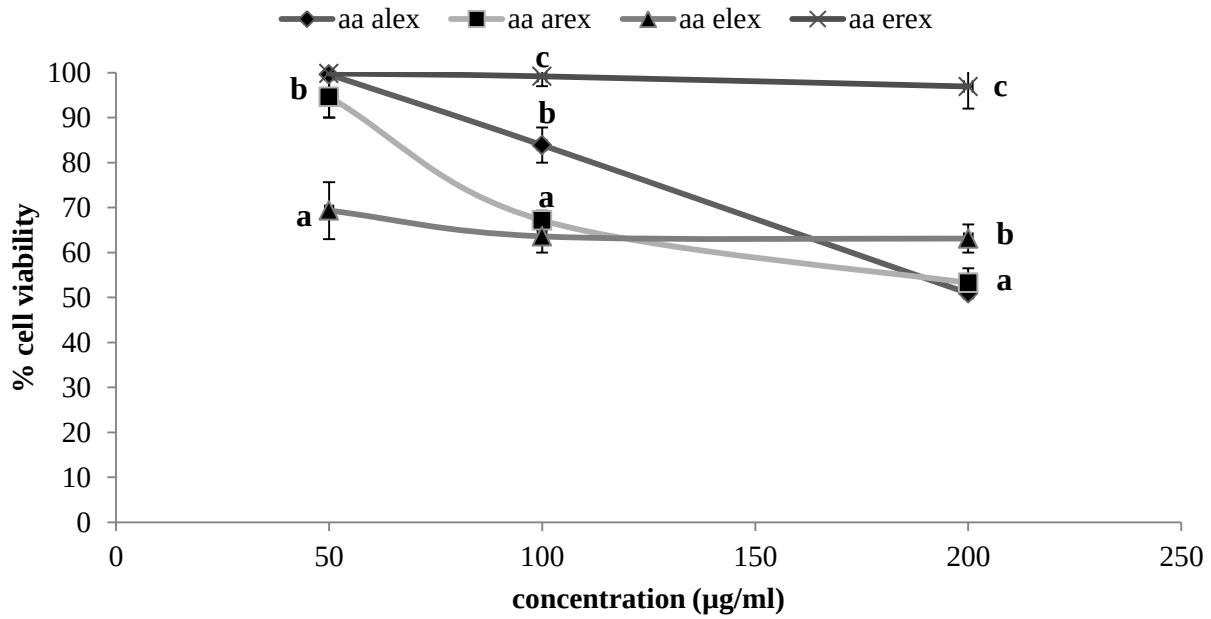


**Fig. 3.6**: Cytotoxicity activity *A.antunesiana* extracts on HEK 293 kidney cells lines as confirmed by MTT cell proliferation assay. Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD values of triplicate determinations. [a-c]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range post hoc test, P<0.05).

**Table 3.8**: $IC_{50}$ values for cytotoxicity activity of *A.antunesiana* extracts on HEK 293 kidney cells.

| **Name of extract** | **Cytotoxicity $IC_{50}$ values in (µg/mL)** |
|---|---|
| **Aa elex** | 306.58 ± 171.22 [d] * |
| **Aa alex** | 223.05 ± 25.40 [c] |
| **Aa erex** | 6.14 ± 325.01 [a] * |
| **Aa arex** | 206.71 ± 13.90 [b] |

Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. The * represent the units of these values which are in mg/ml. Data are presented as mean ± SD values of triplicate determinations. [a-d]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05).

### 3.2.6 Discussion

One treatment option for diabetes mellitus is by decreasing postprandial hyperglycemia and this could be achieved by delaying the digestion of carbohydrates in the digestive tract through inhibiting carbohydrate hydrolysing enzymes (α-amylase and α-glucosidase). By inhibiting these key enzymes, minimal amounts of glucose would be absorbed into the blood stream, hence the plasma glucose will not spike after a meal (Ferrannini *et al*., 2004).

Present study shows that *A.antunesiana* root extracts moderately inhibited α-amylase and significantly inhibited α-glucosidase activity as evidently shown by the $IC_{50}$ values (Fig. 3.8). This mimics an effective T2DM drug model which has to be strong potent inhibitor of intestinal α-glucosidases and mildly inhibits pancreatic α-amylase (Krentz and Baile, 2005)**.** This approach however has been reported to cause stomach discomfort due to fermentation of undigested carbohydrates materials by bacterium in the colon. The main objective of the *in vitro* α-glucosidase and *in vitro* α-amylase inhibitory assays were to provide get more insight in the *in vitro* potential for the potential inhibition of α-glucosidase and α-amylase and to produce a stronger biochemical justification for further possibility of *in vivo* studies on extracts of *A.antunesiana* based on the observed data.

In a previous study, we found that the *A.antunesiana* ethanol root extract contains mainly phenolic compounds (catechol, pyrogallol and hydroxyquinol and resorcinol), coumarin and triterpenoids (α - and β-amyrin) as the major phytochemical components. Besides having the highest antioxidant activity, the ethanol root extract was found to be effective α-glucosidase and α-amylase inhibitor at least in this *in vitro* study which could be linked to the above mentioned phenolic compounds. This is because phenolic compounds were reported to be effective α-glucosidase and α-amylase inhibitors (de Sousa *et al*., 2004, Hanamura *et al*., 2005;Thilagam *et al*., 2013). Based on these observations, the observed inhibitory activity of *A. antunesiana* ethanol root extract could be linked due to one or more of the above-mentioned compounds present in the extracts. Furthermore, some of the compounds found in this extract such as phenolics, coumarin and triterpenoids were reported as being effective inhibitors of α- amylase and α-glucosidase in a recent study (Thilagam *et al*., 2013). Hence, our *in vitro* data suggest the potential anti-diabetic activity of *A.antunesiana* ethanol root extract via inhibiting two major carbohydrate metabolizing enzymes, α-glucosidase and α-amylase.

In order to ascertain the safety of the extracts, the cytotoxicity activities of *A.antunesiana* extracts were investigated and we found out that aqueous leaf and aqueous root extracts showed a significant cytotoxicity activity as confirmed in Fig. 3.6 by the reduction in cell viability by approximately 51 and 53% respectively at 200 µg/ml concentration. However the ethanol root extract did not significantly decrease the viability of cells. Therefore, it necessitates the further investigation of the aqueous extracts to identify the responsible compounds for the observed toxic activity and further assess their chemotherapeutical properties. Also further aiming to identify the bioactive compounds of the ethanol root extracts so as to separate them and realise the full therapeutic potentials of the bioactive components.

Based on the results presented in this section, it can be concluded that ethanolic root extract of *A.antunesiana* exerts an inhibitory effect on α-glucosidase and α-amylase and also shows to be relatively non-toxic at tested concentrations. Furthermore, the results displayed by the ethanol root extract are interesting enough to be used in *in vivo* models. The obtained data further agree with logic behind the traditional use of plants in medicine based on their inhibitory activity of glucose absorption in the gut.

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# CHAPTER 4

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***In vitro* antioxidant, anti-hypergycemic and cytotoxicity activity of *Cissus cornifolia* extracts.**

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# 4.1 *In vitro* antioxidant activity and GC-MS analysis of the ethanol and aqueous extracts of *Cissus cornifolia* (Baker) Splanch (Vitaceae) parts

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**Running title:**

Anti-oxidative potential of *Cissus cornifolia*.

### 4.1.1 Preface

This chapter has been accepted for publication in the Acta Poloniae Pharmaceutica recently.

### 4.1.2 Abstract

The study was intended to explore the antioxidant potential and phytochemical content of the ethanol and aqueous extracts of the leaf and root samples of *Cissus cornifolia* (Baker) Splanch (Vitaceae) across a series of four in vitro models. The results showed that all the extracts had reducing power ($Fe^{3+}$- $Fe^{2+}$), and DPPH, hydroxyl and nitric oxide radical scavenging abilities to varying extents. However, the ethanol root extract had more potent antioxidant power in all the

experimental models than other extracts and possess a higher total phenol content of 136.1± 6.7 mg/g. The GC-MS analysis of the aqueous and ethanol extracts of the roots indicated the presence of the common aromatic phenolic compounds, pyrogallol, resorcinol and catechol, a fatty acid, n-hexadecanoic acid and an aldehyde, vanillin. Data from this study suggest that both the leaves and roots of *C. cornifolia* possessed anti-oxidative activities with the best anti-oxidant activity being exhibited by the ethanolic extract of the root. The antioxidant properties of the root extracts can be attributed to the phenolic compounds present in the extracts.

**Key words**: antioxidant, *Cissus cornifolia*, in vitro, polyphenols.

### 4.1.3 Introduction

The biochemical state of the body, when there is an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage, is termed oxidative stress (OST). Oxidative stress has been implicated in the pathogenesis of a number of diseases such as atherosclerosis, Parkinson's, heart failure, myocardial infarction, Alzheimer's disease, fragile X syndrome, chronic fatigue syndrome and diabetes (Calabrese *et al*., 2012).

The deleterious effects of oxidative stress in a number of metabolic chronic disorders has prompted scientists to search for antioxidative compounds that can impede the oxidation of biomolecules in a chain reaction, which could be vital in the therapy and prevention of many metabolic disorders (Wright *et al*., 2006). The synthetic antioxidative agents, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tertiobutylhydroxytoluene exhibit potent free radical scavenging effects but they induce liver and kidney dysfunction and have also been reported to be carcinogenic in laboratory animals (Saito *et al*., 2003; Subhasree *et al*., 2009; Djeridane *et al*., 2010). Thus, there is a need to identify and utilize more antioxidants of natural origin, which can combat the deleterious effects of free radicals and other biological oxidants (Djeridane *et al*., 2010).

Plants have a long history of use in medicinal applications, especially in Sub-saharan Africa where access to medical supplies, pharmaceutics and medical doctors are limited. The uses of these medicinal plants have been well documented over the years and their use, especially in the rural areas of African countries is quite popular (van Wyk *et al*., 2008). Based on this, research

activities on medicinal plants, especially on the phytochemistry and bioactivity of medicinal plants have been stimulated in order to develop alternative therapies for a number of diseases (Musa *et al*., 2008). Recently the screening of medicinal plants and plant products potential antioxidant and antidiabetic has received significant attention due to an increasing fear for unsafe drugs (Aliyu *et al*., 2012).

*Cissus cornifolia* (Baker) Splanch (Vitaceae), commonly called the "Ivy grape" is indigenous to Zimbabwe. It is locally called ''Mudzambiringa'' and ''Idebelebe'' by the Shona and Ndebele-speaking Zimbabweans respectively. *Cissus* is a genus of approximately 350 species. Most of the members of this genus have a wide array of uses in African traditional medicine (Wild *et al*., 1969). *C. cornifolia*, commonly found in Zimbabwe, is traditionally used by the Shona speaking people as a remedy for gonorrhea while the leaf-sap is used among the Tanganyika as a sedative in cases of mental derangement; the root-decoction is also used for malaria, septic tonsil, diabetes, cardiac problems and pharyngitis (Burkill, 2000). Currently, there have been no phytochemical or biological activity studies on *C. cornifolia.* Phytochemical studies on other species of *Cissus* have revealed the presence of glycosides, flavonoids, saponins, steroids, terpenoids and tannins (Jimoh *et al*., 2013; Musa *et al*., 2011; Jainu *et al*., 2006; Varadarajan *et al*., 2008).

Bioactivity and isolation of various compounds have been carried out in a number species from the genus *Cissus* and had shown impressive results. *Cissus quadrangularis* Linn. Wall. Ex which is commonly used as a food supplement in India, was evaluated for its protective effects against neutrophils (Jainu *et al*., 2006). Jainu and Devi (2003) reported that *Cissus quadrangularis* has antimicrobial, antiulcer, antioxidative and cholinergic activity as well as potent fracture healing properties and a beneficial effect on cardiovascular diseases. In addition, the same group reported cytoprotective properties of the methanolic extract of the same plant (Jainu *et al*., 2004). Attawish *et al*. (2002) isolated Vitamin C, β-carotene, two asymmetric tetracyclic triterpenoids, β-sitosterol, α-amyrin, α-amyrone from *Cissus quadrangularis* which they accredited to the observed activities and Potu *et al*. (2010) also reported several phytochemical constituents, such as ascorbic acid, flavonoids, and triterpenoids in *Cissus quadrangularis*.

*Cissus sicyoides* L, has been widely used in folk medicine against stomachache and indigestion (Asprey and Thornton., 1954) and has been reported to treat diabetes, pain, inflammation,

rheumatism, abscesses, muscle inflammation, convulsions, epilepsy, stroke and hypertension (Ferreira *et al*., 2008). Ferreira *et al*. (2008) also reported a coumarin glycoside, coumarin sabandin, flavonoids, steroids, sitosterol and hydrolysable tannins from the plant. Cissus populnea has been reported as a food supplement in Nigeria (Ibrahim and Dawes, 2000), however there are no reports on the phytochemistry of the plant in the literature. Ojekale *et al*. (2007) reported *Cissus populnea* to have a myriad of uses as a medicinal agent globally.

**Fig. 4.1:** Picture of *Cissus cornifolia* plants. Picture taken from Chivake River, Mrewa, Zimbabwe

This study was undertaken to investigate the ethnomedicinal efficacy of the leaf and root ethanol and aqueous extracts of the plant as potential sources of therapeutic agents which can be of use in ameliorating oxidative stress related parameters using various models in vitro. We also subjected the extracts to GC-MS analysis in order to partially explore the phytochemistry of the plant.

## 4.1.4 Materials and Methods

Please refer to the section **2.1 – 2.4** and **2.7** in pages (**43 – 54**) for detailed materials and methods.

### 4.1.5 Results

**Table 4.1:** Percentage recovery (g/g) and total phenolic content of various solvent extracts of *C. cornifolia* parts

| Samples | % Recovery (g/g) | Total polyphenol (mg/g GAE) |
|---|---|---|
| **Leaves** | | |
| EtOH | 2.70 | $52.9 \pm 12.5^{a}$ |
| Aqueous | 5.91 | $89.9 \pm 7.40^{b}$ |
| **Root** | | |
| EtOH | 1.69 | $136.1 \pm 10.6^{d}$ |
| Aqueous | 7.60 | $106.0 \pm 23.6^{c}$ |

Data are presented as mean ± SD values of triplicate determinations. $^{a-d}$Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The percentage recovery (g/g) obtained from various extracts (**Table 4.1**) collected from the plant indicated that the highest recovery was obtained in the aqueous extracts. All extracts were found to possess a high total phenolic content. However, the highest total phenolic content was recorded in the ethanol extract of the roots (136.1 ± 10.6 mg/g GAE) (Table 4.1).

**Table 4.2** shows the total reducing power of the ethanol and aqueous extracts of *C. cornifolia* parts. All the extracts of *C. cornifolia* showed an ability to donate electrons to convert $Fe^{3+}$-$Fe^{2+}$ as indicated by the concentration dependent increase in the percentage reducing power. The ethanol extract of the root had a significantly ($P<0.05$) higher total reducing power than other extracts.

**Table 4.2**: Percentage total reducing power (GAE) of solvent extracts from various parts of *C. cornifolia*

| | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| Extracts | 15 | 30 | 60 | 120 | 240 |
| **Leaves** | | | | | |
| EtOH | 9.81 ± 1.30[a] | 14.12 ± 2.45[a] | 21.37 ± 1.33[b] | 28.59 ±1.14[a] | 34.00 ± 9.07[a] |
| Aqueous | 4.65 ± 3.39[a] | 10.79 ± 4.54[a] | 14.51 ± 0.88[a] | 29.15 ± 5.69[a] | 30.71 ± 1.00[a] |
| **Root** | | | | | |
| EtOH | 29.75 ± 1.12[c] | 45.75 ± 2.66[c] | 52.80 ± 0.45[d] | 75.22 ± 2.39[c] | 93.09 ± 1.05[c] |
| Aqueous | 15.95 ± 2.02[b] | 30.18 ± 0.20[b] | 40.69 ± 0.63[c] | 45.71 ± 1.80[b] | 75.51 ± 1.10[b] |
| **Standard** | | | | | |
| Ascorbic acid | 76.83 ± 4.92[d] | 80.24 ± 3.00[d] | 90.26 ± 5.51[e] | 91.08 ± 5.43[d] | 93.60 ± 6.21[c] |

Data are presented as mean ± SD values of triplicate determinations. [a-e] Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$.

**Fig. 4.2** shows the DPPH radical scavenging activities of the leaf (A) and root (B) extracts of *C.cornifolia* parts. All the extracts showed an ability to quench DPPH free radicals as indicated by the concentration dependent increase in the percentage inhibition. The root and leaf ethanol extracts had a consistently higher DPPH radical scavenging activity, which is concentration dependent (**Fig. 4.2**).

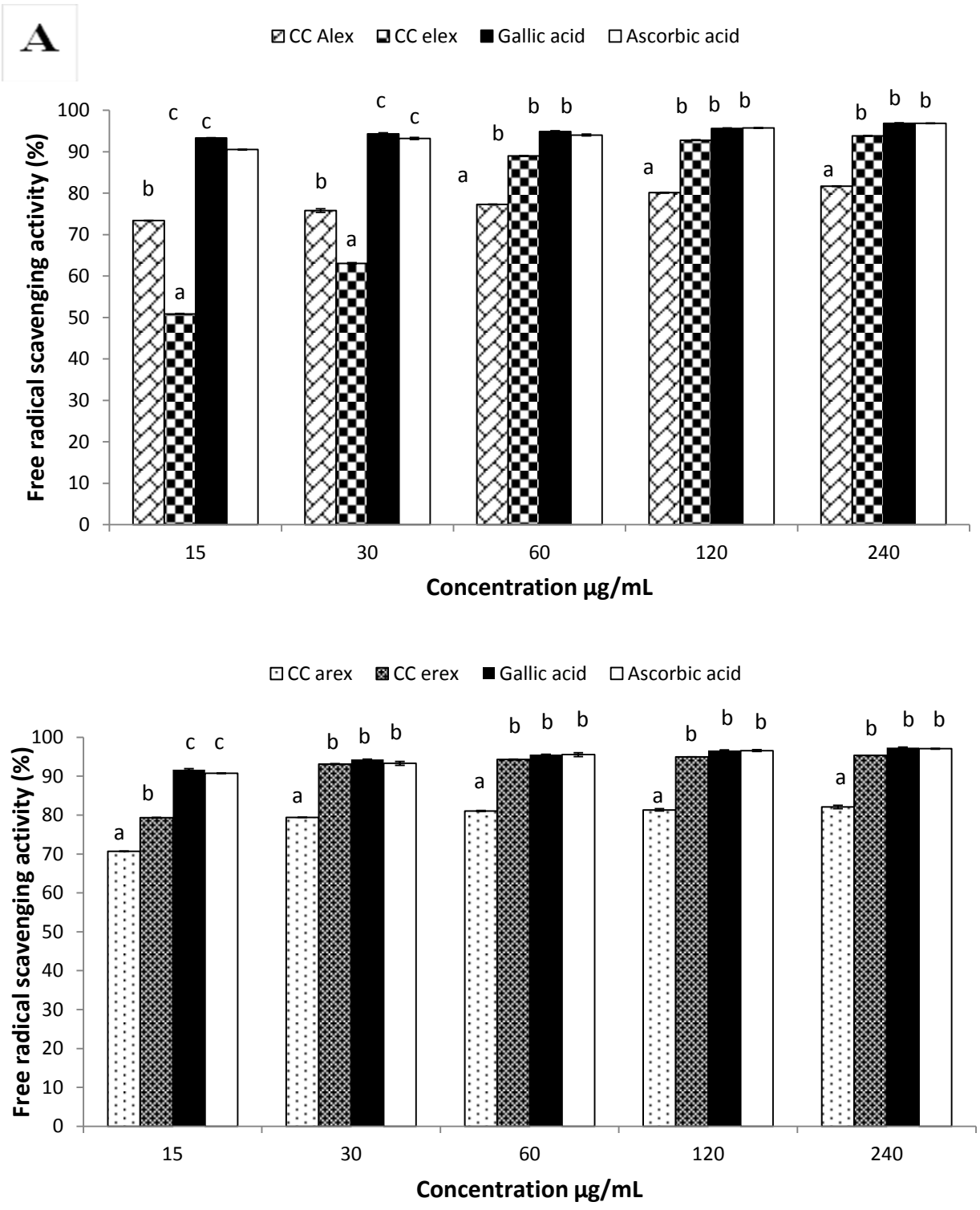


**Fig. 4.2**: DPPH radical scavenging activity of ethanol and aqueous extracts of the leaves (A) and roots (B) of *C. cornifolia*. Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD of triplicate determinations. $^{a-c}$Values with different letters over the bars for a given concentration of each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

The results of the HRS assay indicated that all (ethanol and aqueous) extracts could scavenge hydroxyl radicals generated by Fenton's reaction with the ethanol extracts displaying significantly higher scavenging abilities compared to the aqueous extracts and the standards used (**Table 4.3**). It is also notable that the ethanol extract of the root consistently demonstrated a significantly higher ($P<0.05$) HRS activity than all other extracts.

**Table 4.3**: Percentage hydroxyl radical scavenging activity of extracts from the root and leaves of *C. cornifolia*

| | **Concentration (µg/ml)** | | | | |
|---|---|---|---|---|---|
| | 15 | 30 | 60 | 120 | 240 |
| *Extracts* | | | | | |
| **Leaves** | | | | | |
| Ethanol | $38.96 \pm 1.2^{c}$ | $55.41 \pm 1.3^{e}$ | $70.34 \pm 0.3^{d}$ | $78.57 \pm 0.1^{d}$ | $83.54 \pm 0.7^{d}$ |
| Aqueous | $24.98 \pm 1.0^{b}$ | $27.89 \pm 1.2^{b}$ | $43.51 \pm 4.8^{c}$ | $48.63 \pm 3.3^{c}$ | $54.12 \pm 2.2^{b}$ |
| **Root** | | | | | |
| Ethanol | $27.51 \pm 1.7^{b}$ | $47.40 \pm 1.3^{d}$ | $64.71 \pm 0.2^{d}$ | $88.46 \pm 2.0^{e}$ | $92.96 \pm 2.1^{e}$ |
| Aqueous | $26.82 \pm 1.3^{b}$ | $20.42 \pm 1.6^{a}$ | $32.13 \pm 2.0^{b}$ | $51.50 \pm 0.3^{c}$ | $69.90 \pm 5.6^{c}$ |
| **Standards** | | | | | |
| Ascorbic acid | $14.50 \pm 2.20^{a}$ | $17.35 \pm 3.10^{a}$ | $21.85 \pm 0.10^{a}$ | $26.80 \pm 0.70^{a}$ | $30.30 \pm 1.20^{a}$ |
| Gallic acid | $32.00 \pm 0.60^{c}$ | $35.57 \pm 2.30^{c}$ | $36.75 \pm 1.80^{b}$ | $38.90 \pm 1.10^{b}$ | $40.20 \pm 0.20^{a}$ |

Data are presented as mean ± SD values of triplicate determinations. $^{a-e}$Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$

**Table 4.4** presents the NO inhibition activities of ethanol and aqueous extracts of *C.cornifolia* leaves and roots. Root extracts (ethanol and aqueous) were found to exhibit better NO inhibition activity but the ethanol extract of the root demonstrated a higher NO inhibition effect than other extracts. Furthermore, the root extracts possessed lower $IC_{50}$ values of 24.86 and 36.33 µg/mL respectively, than the corresponding leaf extracts in these plant parts (**Table 3.5**).

**Table 4.4**: Percentage nitric oxide (NO) scavenging activities of extracts from various parts *C. cornifolia*

| *Extracts* | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| | **15** | **30** | **60** | **120** | **240** |
| **Leaves** | | | | | |
| Ethanol | $18.75 \pm 2.5^{a}$ | $27.48 \pm 1.9^{b}$ | $28.24 \pm 0.7^{b}$ | $43.51 \pm 1.4^{b}$ | $48.85 \pm 0.5^{b}$ |
| Aqueous | ND | $11.19 \pm 0.6^{a}$ | $12.91 \pm 1.0^{a}$ | $16.28 \pm 0.7^{a}$ | $22.95 \pm 0.7^{a}$ |
| **Root** | | | | | |
| Ethanol | $42.23 \pm 1.4^{b}$ | $44.52 \pm 0.9^{c}$ | $57.58 \pm 1.0^{c}$ | $83.11 \pm 2.2^{d}$ | $91.18 \pm 1.5^{d}$ |
| Aqueous | $46.56 \pm 2.4^{b}$ | $50.63 \pm 1.0^{c}$ | $54.7 \pm 0.7^{c}$ | $63.15 \pm 0.1^{c}$ | $70.48 \pm 0.8^{c}$ |
| **Standards** | | | | | |
| Ascorbic acid | $51.44 \pm 0.05^{c}$ | $56.94 \pm 0.90^{c}$ | $58.37 \pm 1.40^{c}$ | $62.44 \pm 0.70^{c}$ | $77.22 \pm 1.20^{c}$ |
| Gallic acid | $43.06 \pm 9.20^{b}$ | $47.36 \pm 0.70^{c}$ | $49.76 \pm 0.70^{c}$ | $59.33 \pm 3.20^{c}$ | $64.59 \pm 6.20^{c}$ |

Data are presented as mean ± SD values of triplicate determinations. [a-d]Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$); ND not determine

**Table 4.5:** $IC_{50}$ values of various solvent extracts of *C. cornifolia* parts in different anti-oxidative models

| | IC 50 values in µg/ml | | |
|---|---|---|---|
| Samples | DPPH | HRS | NO |
| **Leaves** | | | |
| Ethanol | 11.02 ± 3.44 [a] | 26.63 ± 4.33 [a] | 286.96 ± 14.25 [d] |
| Aqueous | 47.46 ± 9.10 [b] | 160.91 ± 12.54 [d] | 589.14 ± 23.55 [e] |
| **Root** | | | |
| Ethanol | 6.83 ± 1.22 [a] | 22.94 ± 5.22 [a] | 24.86 ± 3.88 [b] |
| Aqueous | 10.54 ± 2.01 [a] | 123.11 ± 10.99 [c] | 36.33 ± 4.11 [c] |
| **Standards** | | | |
| Ascorbic acid | 5.01 ± 0.76 [a] | 64.65 ± 7.78 [b] | 15.13 ± 3.22 [a] |
| Gallic acid | 5.62 ± 2.34 [a] | 6.30 ± 205.67 [e]* | 41.68 ± 2.90 [c] |

The * represent the units of these values which are in mg/ml. Data are presented as mean ± SD values of triplicate.

**Table 4.6:** Compounds identified from the root of *C. cornifolia* by GCMS

| Name of compound | Retention time (mins) | Percentage match (%) | Molecular mass (g/mol) |
|---|---|---|---|
| **Ethanol extract** | | | |
| Hydroquinone | 10.71 | 86 | 110 |
| Resorcinol | 10.83 | 99 | 110 |
| Vanillin | 12.56 | 92 | 152 |
| n-Hexanoic acid | 18.58 | 91 | 256 |
| **Aqueous extract** | | | |
| Catechol | 10.12 | 95 | 110 |
| Resorcinol | 11.25 | 97 | 110 |
| Pyrogallol | 12.55 | 72 | 126 |
| **Ethanol extract** | | | |
| Hydroquinone | 10.71 | 86 | 110 |

Identified compounds of EtOH and aqueous extracts of different parts of *C.cornifolia* by GC-MS, from all extracts of ethanol and aqueous aromatic compounds were identified are presented in **Table 4.6**. Three polyhydroxylated phenols, pyrogallol, catechol and resorcinol were extract respiration.

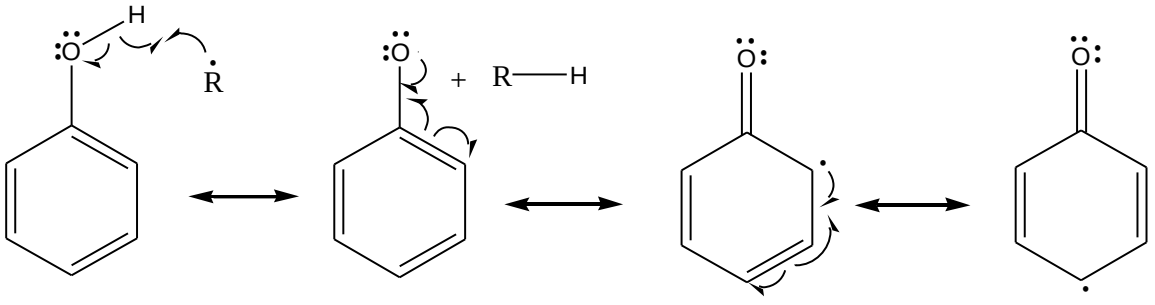


**Figure 4.3**. Hydrogen radical donation to a reactive radical species and stabilization of the resultant radical.

### 4.1.6 Discussion

*Cissus cornifolia* has been reported to have a variety of uses among which, the root decoction is used by traditional healers and herbalist to treat and manage a vast array of non-metabolic chronic disorders and ailments such as diarrhea and other stomach ailments, back pain, sore throat, wounds, diabetes and cardiac problems in the Mrewa district in Zimbabwe (personal communication with traditional healers).

Preliminary investigations on the *in vivo* neuropharmacological activity of the methanolic leaf extract as well as its hypoglycemic activity on normoglycemic rats have been reported (Musa *et al*., 2008; Jimoh *et al*., 2013). Nevertheless, according to our current knowledge, there is no report on detailed anti-oxidative activities from any part of the plant or its phytochemistry. A set of *in vitro* assays were employed to cover most possible known mechanisms by which different antioxidants operate to inhibit oxidative chain reactions.

The results from all the experimental models (total reducing power ($Fe^{3+}$-$Fe^{2+}$ ), DPPH, hydroxyl radical and nitric oxide reducing ability) indicated that all the different parts of *C. cornifolia* extracted in ethanol and water possess antioxidant potential in a concentration dependent manner to varying extents. The ethanolic root extract has consistently shown to have exceptionally high antioxidant ability and the recorded high phenolics content (136.1 ± 10.6 mg/g GAE), which supported the observed high activity and effectiveness of the extract in the *in vitro* assays.

To identify the responsible phytochemicals, we carried out GC-MS analysis of the aqueous and ethanol extracts of the leaves and roots. Only long chain aliphatic compounds were identified in the ethanol and aqueous extract of the leaves. Palmitic acid, arachidic acid and nonacosane were identified by comparing their MS spectra to those of standard spectra from the NIST library. While no phytochemicals could be identified in the leaf extracts of the plant, the root extracts contained polyphenol compounds, known to be potent antioxidant compounds and is most likely responsible for the antioxidant activity in the root extract of the plant. Two isomers of benzenediol (hydroquinone and resorcinol) were found in the ethanol extract of the roots.

It is highly probable that the polyphenols in the roots are the reason why the roots are more active as antioxidants than the leaves. The mechanism by which these compounds act as radical scavengers is via the transfer of a hydrogen atom to a radical species, thereby creating a radical

charge on the polyphenol, which is delocalized on the aromatic ring stabilizing the radical charge (Fig. 4.3). The radical benzene molecule can be quenched by reacting with other radical species.

In conclusion, the results of this study suggest that the ethanolic extracts of leaf and root have strong anti-oxidative effects which might be contributed by some major bioactive active compounds such as hydroquinone, resorcinol, vanillin and n-hexanoic acid. The anti-oxidative effects of the root ethanolic extract were better than the leaf ethanolic extract which can be used to investigate the in vivo anti-oxidative and anti-diabetic effects in the animal model of type 2 diabetes. Additionally, findings of this study further support the use of the root and leaf extracts by traditional healers as an anti-diabetic medicine.

### 4.1.7 Post-script analysis

*C. cornifolia* extracts were further subjected to *in vitro* α-amylase, α-glucosidase inhibitory and cytotoxicity assays and the results were presented in the following section.

# 4.2 *In vitro* α-amylase and α-glucosidase inhibitory and cytotoxicity activities of ethanol and aqueous extracts of *Cissus cornifolia* Planch parts

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**Running tittle:**

Antidiabetic and cytotoxicity activity of *Cissus cornifolia* parts.

### 4.2.1 Preface

This paper has not yet been submitted for publication.

### 4.2.2 Abstract

Hyperglycemia being the hallmark of type 2 diabetes mellitus, preventing it will go a long way in managing diabetes mellitus and its late complications. One therapeutical approach is by decreasing postprandial hyperglycemia this is achieved by retarding glucose absorption through inhibiting carbohydrates digesting enzymes (α-amylase and α-glucosidase). *Cissus cornifolia* extracts were investigated for α-amylase and α-glucosidase inhibitory activity *in vitro*. The root extracts (ethanol and aqueous) all had mild α-amylase inhibition activity and strong α-glucosidase for ethanol root and aqueous root mild inhibition power also supported by $IC_{50}$ values (22.75 ± 1.23 and 33.70 ± 3.75 µg/ml) for α-amylase and (2.81 ± 0.97 and 37.48 ± 2.35

µg/ml) for α-glucosidase. The ethanolic extracts shows to be relatively non-toxic at tested concentrations on the human embryonic kidney (HEK 293) cell lines as confirmed by the MTT assay. However aqueous extracts (leaf and root) were cytotoxic at concentrations above 50 µg/ml. Data from this study suggest that the ethanolic extracts of roots of *C. cornifolia* possessed α-amylase, α-glucosidase inhibitory activity *in vitro* and did not cause significant cell death on the tested concentrations. The inhibition activity observed is strongly thought to be as a result of the identified polyphenols. The observed trend necessitates further investigations on the ethanol root extract *in vivo.*

### 4.2.3 Introduction

Diabetic cases have increased tremendously and expected to double in the next decade (IDF, 2011; David *et al*., 2011), notably its occurrence in the developing countries where people have adopted high calorie westernized diets with lack of physical activities. Non-insulin-dependent diabetes mellitus (NIDDM) commonly referred to as type 2 diabetes contributes approximately 90-95% of all diabetes cases (Wild *et al.*, 2004). It is an endocrine system dysfunction caused by the diminished secretion of insulin by the pancreatic β-cells of the islets of Langerhans or by reduced sensitivity of cells to insulin action due to excessive absorption of glucose and failure to regulate blood glucose levels which leads to hyperglycemia (Iwamoto, 1995; Shaiq *et al*., 2002).

Long-term diabetic complications have been strongly linked to hyperglycemia. Since the major source of glucose is dietary carbohydrates, hence the inhibition of key carbohydrates digesting enzymes such as α-amylase and α-glucosidase, would be vital in preventing late diabetic complications. This is because inhibitors of these enzymes (α-amylase and α-glucosidase) delays the digestion of carbohydrates, thereby prolongs the overall carbohydrate digestion time and reduces the rate of glucose absorption from the gut and finally, lowers the postprandial rise in blood glucose. Therefore, inhibition of α-amylase and α-glucosidase is a key in managing and treatment of noninsulin-dependent diabetes (Shaiq *et al.*, 2002, Bischoff, 1994)

Conventionally, the control of blood glucose level is achieved by using available oral hypoglycaemic agents and insulin all of synthetic origin, however all these have been reported to have limited efficacy and to pose undesirable side effects (Shaiq *et al*., 2002; Reuser and Wisselaar, 1994). Numerous plant-derived compounds which mimic the action of oral

hypogycaemic agents such as voglibose, miglitol and acarbose have been isolated (Yoshikawa *et al*., 1998; Nishioka *et al*., 1998,; Ortiz-Andrade *et al*., 2007; Shirwaikar *et al*., 2005) and prove to be effective in inhibiting carbohydrates digesting enzymes, hence more search of such plant-derived compounds is required. Given that plant-derived products have shown impressing potentials such that they influence the discovery of some current conventional medicines being used at present (Shirwaikar *et al*., 2005). Unfortunately, some reports indicated the presence of some potential toxic and carcinogenic (Fennell *et al*., 2004) agents in some of these plants which makes them unsuitable for therapeutic applications. It is therefore of the utmost importance to intensively investigate the potential cytotoxic activity in order to validate the safety and continued use of medicinal plants. It has been documented that some plants extracts do have bioactivity however it is cancelled by their cytotoxicity, hence such scenarios need to be expansively evaluated to assess the overall efficacy of the plant extract, also to properly inform on how much of a certain plant extract is safe for therapeutical thereby avoiding overdosing and poisoning body organs.

*Cissus cornifolia* (Baker) Planch (Vitaceae) commonly called the "Ivy grape" is indigenous to Zimbabwe. Locally, it is called ''Mudzambiringa'' and ''Idebelebe'' by the Shona and Ndebele-speaking Zimbabweans respectively. The plant is traditionally used by the Shona speaking people as a remedy for gonorrhea when taken with native natron while the leaf-sap is used among the Tanganyika of Tanzania as a sedative in cases of mental derangement; the root-decoction is also used for malaria, septic tonsil, diabetes, cardiac problems and pharyngitis (Burkill, 2000). Our survey of the medicinal use of *C. cornifolia* in Mrewa District, Zimbabwe also revealed that its roots are used to treat diabetes mellitus among other ailments (personal communications with traditional healers).

Bioactivity and isolation of various compounds have been carried out in a number species from the genus *Cissus* and had shown impressive results. *Cissus quadrangularis*, *Cissus sicyoides* were explored and various phytoconstituents were identified which include: Vitamin C, β-carotene, two asymmetric tetracyclic triterpenoids, β-sitosterol, α-amyrin, coumarin glycoside, coumarin sabandin, flavonoids, steroids, sitosterol and hydrolysable tannins (Attawish *et al*., 2002; Ferreira *et al.*, 2008). These compounds have been all report to play a major role in the medicinal value of these species (Ferreira *et al.*, 2008).

At present, not much scientific information on *C. cornifolia* exists in the literature apart from preliminary reagent-based phytochemical analysis which revealed that the plant possesses glycoside, flavonoids, saponins, steroids, terpenoids, tannins (Jimoh *et al.*, 2013; Musa *et al.*, 2008; Mariana *et al.*, 2008; Jainu *et al.*, 2006 and Varadarajan *et al.*, 2008). According to the GC/MS results from our earlier study polyphenolic compounds such as pyrogallol, resorcinol and catechol, vanillin (aldehyde) and long chain fatty acids were identified as phytochemicals present and possibly responsible for the antioxidant activity observed.

Polyphenols such as the ones we identified using GC/MS to be present in the extracts of *C.cornifolia* e.g. pyrogallol, resorcinol and catechol have not only been reported to possess antioxidant activities but the ability of inhibiting carbohydrate-hydrolyzing enzymes(α-amylase and α-glucosidase), henceforth can prevent hyperglycemia (Hanamura *et al*., 2005, Thilagam *et al*., 2013). However, despite the extensive use of these plant parts in traditional medicine, scientific reports on the biological and pharmacological actions of this plant are limited.

The present study was therefore undertaken to intensively probe the antidiabetic and cytotoxicity activities of the *C.cornifolia* ethanol and aqueous root and leaf extracts as potential sources of nontoxic therapeutical agents which can be of use in achieving normoglycaemia, in order to avoid diabetes and its associated complications, using various scientific models in vitro.

### 4.2.4 Materials and methods

Please refer to the section **2.1 - 2.2** and **2.5 - 2.7** in pages **(43 - 54)** for detailed materials and methods.

### 4.2.5 Results

Fig. 4.5 shows the inhibitory activity of *C.cornifolia* ethanol and aqueous extracts on α-amylase and α-glucosidase respectively. Fig. 4.5A is indicating the ability of ethanol and aqueous root extracts to have significantly higher ($P<0.05$) inhibitory activity on α-amylase than acarbose and all leaf (ethanol and aqueous) extracts do possess inhibition potential which is lower than that of the acarbose. Fig. 4.5B also indicated the ability of root extracts to have better inhibiting activity on α-glucosidase than acarbose. However the ethanol root extract inhibitory activity is significantly higher ($P<0.05$) than all other extracts and acarbose and that of the aqueous extract

is not significantly different from acarbose and all the leaf extracts (ethanol and aqueous) have lower activities than acarbose.

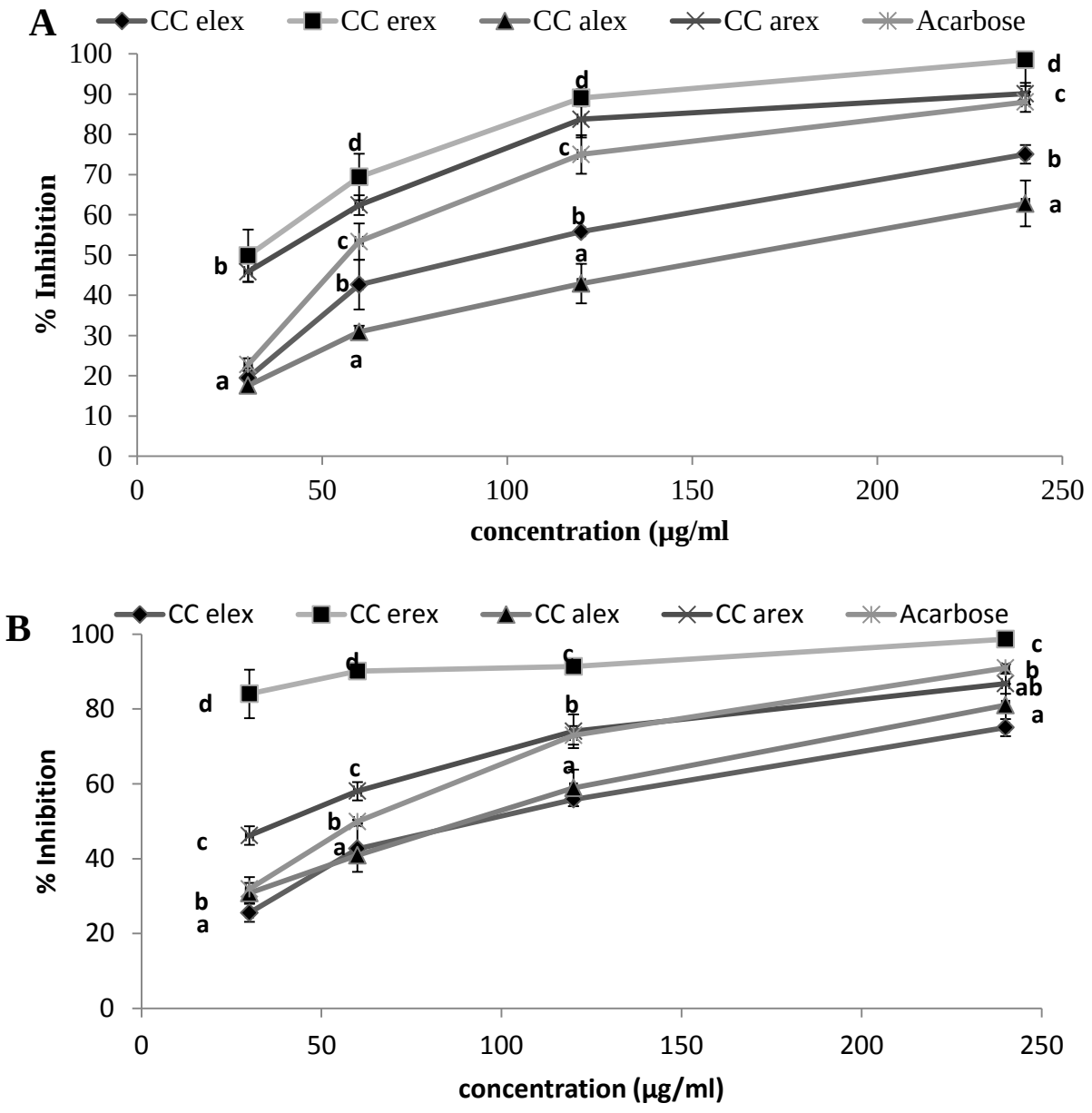


**Fig. 4.5:** A and B = α-amylase and α-glucosidase respectively shows inhibitory activity of *C.cornifolia* aqueous and ethanol extracts. Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD values of triplicate determinations. [a-d] Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05).

**Table 4.7**: $IC_{50}$ values for α-amylase and α-glucosidase inhibition activity of *C.cornifolia* extracts

| | $IC_{50}$ values in (µg/mL) | |
|---|---|---|
| **Name of extract** | **α-Amylase** | **α-Glucosidase** |
| **Cc elex** | 90.78 ± 3.20 [c] | 85.62 ± 5.54 [e] |
| **Cc alex** | 145.24 ±17.90 [d] | 75.31 ± 9.34 [d] |
| **Cc erex** | 22.75 ±1.23 [a] | 2.81 ± 0.97 [a] |
| **Cc arex** | 33.70 ± 3.75 [a] | 37.48 ± 2.35 [b] |
| **Acarbose** | 52.11 ± 0.56[b] | 57.18 ± 3.54[c] |

Data are presented as mean ± SD values of triplicate determinations. [a-e] Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$). Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract

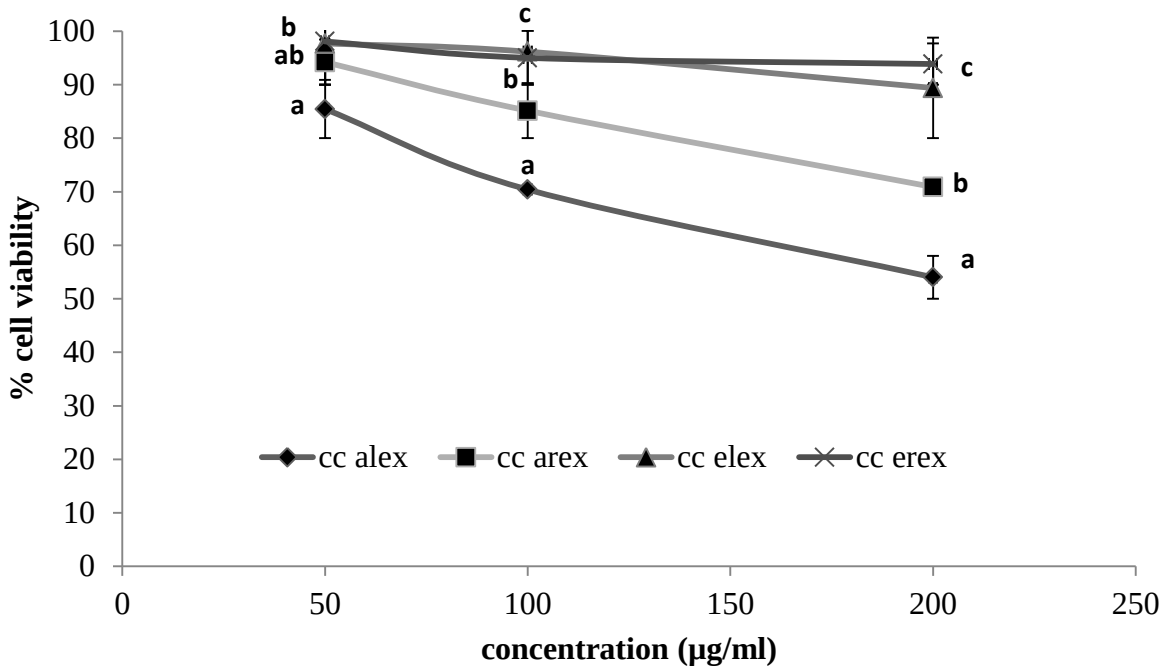


**Fig. 4.6**: Cytotoxicity activity *C.cornifolia* extracts on HEK 293 kidney cells lines as confirmed by MTT cell proliferation assay. Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract. Data are presented as mean ± SD values of triplicate determinations. [a-c] Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05). Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract

The $IC_{50}$ values of the root (ethanol and aqueous) extracts for inhibiting α-amylase and α-glucosidase were significantly lower than acarbose and leaf (ethanol and aqueous) extracts. However, within the root (ethanol and aqueous) extracts, the ethanol extract had a more potent enzyme inhibitory activity than the aqueous extract (Table 4.7). Fig. 4.2 displays the cytotoxicity activity of *C.cornifolia* extracts on HEK 293 kidney cell lines as confirmed by MTT assay. As indicated in Fig. 4.6, *C.cornifolia* ethanol (leaf and root) extracts did not cause any significant decrease in cell viability across all tested concentrations (50 – 200 µg/ml). However, the aqueous (leaf and root) extracts displayed a notable increase in cell death as the concentration of extracts increases. This is also consistent with the high $IC_{50}$ (Table 4.2) values obtained for *C. cornifolia* ethanol root and leaf extracts (2.67 ± 75.44 and 1.63 ± 120.11 mg/ml**)** respectively. All (leaf and root) aqueous extracts showed a significant cytotoxicity activity as confirmed in Fig. 4.6 by the

reduction in cell viability by approximately (50 and 30)% at 200 µg/ml respectively. The leaf extract is the most toxic as indicated by its lowest $IC_{50}$ (Table 4.8) value of 241.29 µg/ml.

**Table 4.8**: $IC_{50}$ values for cytotoxicity activity of *C.cornifolia* extracts on HEK 293 kidney cells.

| Name of extract | Cytotoxicity $IC_{50}$ (µg/mL) |
|---|---|
| **CC elex** | $1.63 \pm 120.11^{c}$ * |
| **CC alex** | $241.29 \pm 21.41^{a}$ |
| **CC erex** | $2.67 \pm 75.44^{d}$ * |
| **CC arex** | $726.67 \pm 35.45^{b}$ |

Data are presented as mean ± SD values of triplicate determinations. [a-d] Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$). Arex= aqueous root extract and Erex= ethanol root extract; Alex= aqueous leaf extract and Elex= ethanol leaf extract

### 4.2.6 Discussion

One therapeutical approach for preventing diabetes mellitus is to retard absorption of glucose through inhibition of key carbohydrates digesting enzymes, α-amylase and α-glucosidase, which are located in the brush borders of the small intestines. Currently, drugs that act by this mechanism include acarbose, miglitol and voglibose (Shaiq *et al.*, 2002). Numerous reports indicates a variety of compounds which had been extracted from medicinal plants possess this ability (Yoshikawa *et al.*, 1998; Nishioka *et al.*, 1998; Ortiz-Andrade *et al.*, 2007; Shirwaikar *et al*., 2005), however, for the first time this study observed the α-amylase and α-glucosidase inhibitory potential of *C. cornifolia*, a plant commonly used for traditional treatment of diabetes in Zimbabwe.

The present study showed that *C.cornifolia* root (ethanol and aqueous) extracts moderately inhibits α-amylase and ethanol root extract significantly inhibit α-glucosidase whilst the aqueous root extract also moderately inhibits α-glucosidase as evidently shown by the $IC_{50}$ in Table 4.8.

According to Krentz and Baile, (2005), better clinical outcome could be derived from specific α-amylase and α-glucosidase inhibitors with a mild inhibitory activity against the α-amylase and strongly inhibiting α-glucosidase and also not cytotoxic to target cells, but still achieving the desired result of delaying postprandial hyperglycemia are ideal potent anti-hyperglycemic compound(s) or drug.

In the present study, *C.cornifolia* leaf extracts to have no significant enzyme inhibition potential. However the root extracts showed inhibition which is greater than acarbose for ethanol root extract however for aqueous extract data are not significantly different from acarbose. We reported earlier the phytoconstituents of *C.cornifolia* root extracts to be mainly polyphenols which include isomers of benzenediol (resorcinol, catechol and hydroquinone), pyrogallol and vanillin (phenolic aldehyde). These compounds are possibly the bioactive components of the extracts. Apart from being effective antioxidants, phenolic compounds and phenol derivatives, have been reported to be potent α-glucosidase and α-amylase inhibitors in some previous and recent studies (de Sousa *et al*., 2004, Hanamura *et al*., 2005, Thilagam *et al*., 2013). So the observed inhibitory activity of the root extracts might be caused by the identified phenolics unlike the leaf extracts, where only long chain aliphatic compounds were identified and no significant inhibitory activity was observed. Thus, based on these results we postulated that the observed inhibitory activity of the *C. cornifolia* ethanolic extract is due to one or more different compounds, especially the phenolics, present in the extracts.

We investigated the cytotoxicity of *C.cornifolia* extracts and we also found out that ethanol extracts did not cause any significant decrease in cell viability however there was notable decrease of viability with aqueous extracts (leaf and root). There were no previous reports on the cytotoxicity activity of *C.cornifolia*. The observed decrease in cell viability is due to HEK 293 cell death; hence, *C.cornifolia* aqueous extracts are cytotoxic at a concentration of 50 µg/ml. Therefore, it necessitates further investigation of *C.cornifolia* extracts to identify the responsible compounds for the observed toxic activity and further assess their chemotherapeutical properties.

The root (ethanol and aqueous) extracts displayed an impressive enzyme inhibition potential as compared to the leaf (ethanol and aqueous) extracts, however the aqueous extracts (leaf and root) caused a significant decrease in cell viability. The aqueous extract shows high enzyme (α-amylase and α-glucosidase) inhibition potential however it is cancelled by the toxic effects it

displayed. So within the root (ethanol and aqueous) extracts the ethanol root extract indicates to be a better anti-hyperglycemic agent. This result could stimulate further investigation of ethanol root extracts in vivo.

Based on the results presented in this study, it can be concluded that ethanolic root extract of *C.cornifolia* exerts an inhibitory effect on α-glucosidase and α-amylase and also shows to be relatively non-toxic at tested concentrations. Furthermore, the results displayed by the ethanol root extract are interesting enough to be used in in vivo models. These results additionally back the traditional use of plants in medicine based on in part, their antioxidant activity and also inhibitory activity of glucose absorption in the gut.

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# CHAPTER 5

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***In vitro* antioxidant, anti-hypergycemic and cytotoxicity activity of *Courbonia glauca* extracts.**

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# 5.1 *In vitro* antioxidant, α-amylase and α-glucosidase inhibition, and cytotoxicity activities of ethanol and aqueous extracts of *Courbonia glauca* (Klotzsch)

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**Running title:**

*In vitro* anti-diabetic effects of *Courbonia glauca* (Klotzsch).

### 5.1.1 Preface:

This section of the thesis is still under preparation into a manuscript to be published shortly.

### 5.1.2 Abstract

The compounds isolated from plant extracts have been reported to have different mechanisms of action behind their anti-diabetic effects both *in vivo* and *in vitro.* Extracts which show one or more of these mechanisms will be valuable for therapeutical applications. In this experiment, such mechanisms were investigated in *Courbonia glauca* ethanol and aqueous extracts. The anti-oxidative activities of the ethanol and aqueous extracts of the leaf and root samples of *C.glauca* were determined across a series of four *in vitro* models. The results showed that all the extracts

had reducing power ($Fe^{3+}$- $Fe^{2+}$), DPPH, hydroxyl and nitric oxide radical scavenging abilities. The ethanol root extract had more potent antioxidant power in all the experimental models and do possess a higher total phenol content of 94.4±7.9 mg/g. The leaf and root ethanol extracts of *C.glauca* was found to possess α-glucosidase inhibitory activity and only the ethanol leaf extract was able to significantly inhibit α-amylase activity. The cytotoxicity of all four extracts of *C. glauca* were also evaluated in HEK293 cell lines. None of the extracts showed significant cytotoxicity in the tested concentration range except for the *C. glauca* aqueous leaf extract which was highly toxic as displayed by its low $IC_{50}$ value of 170.76 µg/ml. Hence three of the extracts are safe for pharmaceutical applications. The GC-MS analysis of the aqueous and ethanol extracts of the roots and leaves indicated that several aromatic phenolic compounds, present in these extracts, could possibly be responsible for the observed activities.

### 5.1.3 Introduction

Diabetes mellitus (DM) is considered as a metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Alberti and Zimmet, 1998). It is a chronic disorder associated with micro- and macrovascular complications and long-term degenerative complications which results in dysfunction of various organs (Lacy and Davie, 1984). The disease is a major health concern globally because it is estimated to affect 285 million people around the world by 2010, with a projected rise to 552 million by 2030 (Gardner *et al.*, 2011).

Out of all the known types of DM, type 2 diabetes (T2D) accounts for approximately 90-95% of all diabetic cases, which is a result of genetic predisposition, life-style and socioeconomic changes mainly characterized by a decrease of physical activity and an increase in high fat intake among other factors (Kriska *et al*., 1993; Dowse *et al*., 1991; Boyce and Swinburn, 1993; Wild *et al*., 2004; IDF Atlas, 5th edition, 2011, Gardner *et al*., 2011). Hyperglycemia is a hallmark of T2D and has been reported to cause oxidative stress through multiple mechanisms. Compelling evidence had demonstrated the key role played by hyperglycemia in the initiation and the facilitation of oxidative stress (OS) (Nishikawa *et al*., 2000; Calabrese *et al*., 2012). Hyperglycemia increased the production of intracellular reactive oxygen species (ROS) through the activation of protein kinase C, generation of advanced glycation end products (AGEs) and

activation of sorbitol pathway which plays a key role in the pathogenesis of diabetic complications (Brownlee, 2000 and 2001; Calabrese *et al*., 2012).

Hence, the devastating effects of hyperglycemia and the resulting OS have compelled the scientific community to search for anti-hyperglycemic and anti-oxidative compounds which can counteract these effects. This will consequently be vital in the therapy and prevention of T2D (Wright *et al*., 2006). One of the most important approaches for the control of hyperglycemia is through the use of α-amylase and α-glucosidase inhibitors (Shirwaikar *et al*., 2005). Inhibition of these key carbohydrates digesting enzymes will ensure a decrease in the postprandial rise in blood glucose level by delaying the digestion of carbohydrates, thereby prolonging the overall carbohydrate digestion time and reducing the rate at which glucose is absorbed from the gut. Thus, inhibition of α-amylase and α-glucosidase is key in the management and treatment of T2D and the prevention of diabetic complications (Shaiq *et al*., 2002, Bischoff, 1994). Based on the above, the identification of a multifunctional compound(s) that acts as an antioxidant and anti-hyperglycemic agent will go a long way in curbing diabetes and its related complications. Such a compound will reduce the costs of synthesizing separate compounds that work on specific mechanism of action. Currently, there are several conventional prescribed α-glucosidase inhibitor drugs which include acarbose, voglibose and miglitol; however, they have been shown to have some undesirable adverse effects such as flatulence, diarrhea and abdominal pain which were hostile to patients (Shaiq *et al*., 2002). This indicates the urgent need for the development of newer alternatives.

Medicinal plants have a long history of being used to treat various ailments and diseases. Some of these plants and plant derived products have influenced the discovery of some current conventional medicines. For example, metformin is an analog of galegine which was extracted from *Galega offinalis* (Jarald *et al*., 2008; Patel, 2012). The use of plants for medicinal purposes still exists in the developing nations, especially in Africa where a wide variety of plants were reported to have many medicinal uses (van Wyk, 2008; Ibrahim *et al*., 2013). This has motivated researchers to keep on working on medicinal plants in order to identify and/or isolate useful bioactive compounds from medicinal plants (Musa *et al*., 2011). Despite the extensive use of plants in alternative medicine, some reports indicated the presence of potentially toxic and carcinogenic agents in some plants, which make them unsuitable for therapeutic applications

(Fennell *et al*., 2004). It is therefore of the utmost importance to intensively investigate the potential cytotoxicity of the plant in order to validate safety for their continued use. It has been documented that although some plant extracts have bioactivity, this is counteracted by their cytotoxicity beyond certain concentration ranges, hence such scenarios need to be evaluated to assess the overall efficacy of the plants (Fennell *et al*., 2004).

*Courbonia glauca* (Klotzsch) Gilg & Bened (Capparaceae) is commonly called the Blue bush-cherry (English), Katunguru (Shona: Zimbabwe) and Muswezu or Soswe (Tonga: Zimbabwe) (Hyde *et al*., 2013). According to a verbal communication survey we conducted in Mrewa and Mutoko districts in Zimbabwe in 2012, the root and leaves of. *C. glauca* were reported to be used to treat cancer, bacterial infections, hypertension and DM as well as wound healing. However, despite the extensive use of these plant parts in traditional medicine, scientific reports on the biological and pharmacological actions of this plant are limited.

The present study was therefore undertaken to intensively probe the antioxidant activities, α-amylase and α-glucosidase inhibitory effects and cytotoxicity of extracts from the root and leaves of *C. glauca*.

### 5.1.4 Materials and methods

Please refer to the section **2.1 – 2.7** in pages (**43 - 54**) for detailed materials and methods.

### 5.1.5 Results

A higher percentage recovery was recorded in the aqueous extracts but the total phenolic content was significantly ($P<0.05$) higher in ethanol extracts with ethanol root extract of *C.glauca* having the highest total phenolic content (Table 5.1).

**Table 5.1:** Percentage recovery (g/g) and total phenolics content of various solvent extracts of *C.glauca* parts

| Samples | % recovery (g/g) | Total polyphenol (mg/g GAE) |
|---|---|---|
| **Leaves** | | |
| EtOH | 2.1 | $80.1 \pm 4.9^{b}$ |
| Aqueous | 7.7 | $64.0 \pm 6.4^{a}$ |
| **Root** | | |
| EtOH | 1.4 | $94.4 \pm 7.9^{c}$ |
| Aqueous | 5.7 | $68.2 \pm 4.0^{a}$ |

Data are presented as mean ± SD values of triplicate determinations. $^{a-c}$ Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

All the extracts of *C. glauca* showed an ability to donate electrons to convert $Fe^{3+}$-$Fe^{2+}$ as indicated by the concentration dependent increase in the percentage reducing power (Table 5.2). However, the ethanol and aqueous extracts of the roots have a higher reducing potential than the leaf extracts at all concentrations.

**Table 5.2**: Percentage total reducing power (GAE) of solvent extracts from various parts of *C.glauca*

| | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| | 15 | 30 | 60 | 120 | 240 |
| Extracts | | | | | |
| **Leaves** | | | | | |
| EtOH | 14.17 ±1.30[a] | 17.24 ± 2.45[a] | 23.59 ±1.33[a] | 28.28 ±1.14[a] | 46.42 ± 9.07[a] |
| Aqueous | 11.21 ± 3.39[a] | 16.03 ± 4.54[a] | 28.12 ± 0.88[a] | 35.44 ± 5.69[a] | 48.48 ±1.00[a] |
| **Root** | | | | | |
| EtOH | 24.41 ± 1.12[b] | 38.55 ± 2.66[b] | 41.40 ± 0.45[b] | 57.93 ± 2.39[b] | 68.24 ± 1.05[b] |
| Aqueous | 18.06 ± 2.02[a] | 28.38 ± 0.20[b] | 44.60 ± 0.63[b] | 53.13 ± 1.80[b] | 62.84 ± 1.10[b] |
| **Standard** | | | | | |
| Ascorbic acid | 76.83 ± 4.92[c] | 80.24 ± 3.00[c] | 90.26 ± 5.51[c] | 91.08 ± 5.43[c] | 93.60 ± 6.21[c] |

Data are presented as mean ± SD values of triplicate determinations. [a-c] Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Fig. 5.1A and 5.1B showing the free radical scavenging activities of the ethanol and aqueous extracts of *C. glauca* roots and leaves clearly indicates the ability of all the extracts to scavenge free radicals proportional to the concentration. The high activity of both the aqueous and ethanol root extracts was markedly evident and being most defined in the aqueous extract.

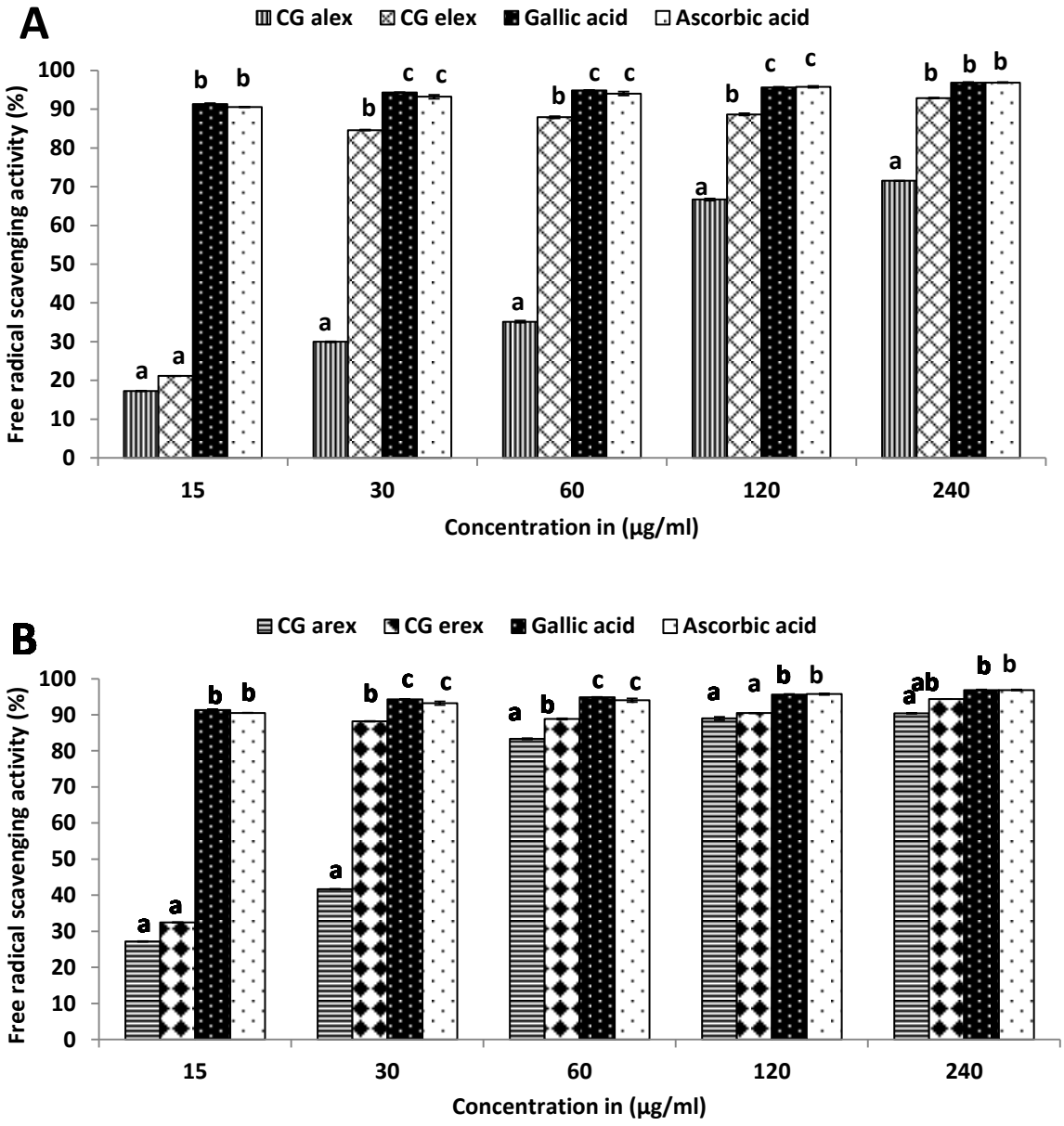



**Figure 5.1A and 5.1B**: DPPH radical scavenging activity of aqueous and ethanol extracts of *C.glauca* leaf and root respectively. **Alex**= aqueous leaf extract; **Elex**= ethanol leaf extract; **Arex**= aqueous root extract; **Erex**= ethanol root extract. Data are presented as mean ± SD of triplicate determinations. $^{a\text{-}c}$Values with different letters over the bars for a given concentration of each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

As indicated in Tables 5.3 and 5.4, the ethanol (leaf and root) extracts displayed significantly higher NO and hydroxyl radical scavenging activity in a concentration dependent pattern as

compared to the aqueous extracts and the two standards (gallic acid and abscorbic acid). $IC_{50}$ values in Table 5.5 also further emphasize the better anti-oxidant activity posed by the ethanol root extract because it had the lowest $IC_{50}$ value across all tested *in vitro* models.

**Table 5.3**: Percentage hydroxyl radical scavenging activity of extracts from the root and leaves of *C.glauca*

| | **Concentration (µg/ml)** | | | | |
|---|---|---|---|---|---|
| | 15 | 30 | 60 | 120 | 240 |
| *Extracts* | | | | | |
| **Leaves** | | | | | |
| Ethanol | 20.56 ± 1.9[c] | 31.60 ± 2.1[c] | 46.11 ± 1.6[d] | 60.82 ± 1.8[d] | 76.62 ± 0.7[c] |
| Aqueous | 7.40 ± 3.7[a] | 7.80 ± 0.6[a] | 11.72 ± 2.3[a] | 20.13 ± 2.0[a] | 33.41 ± 0.7[a] |
| **Root** | | | | | |
| Ethanol | 16.92 ± 3.0[b] | 34.42 ± 0.9[b] | 43.34 ±1.4[d] | 66.55 ± 1.5[d] | 83.65 ±1.6[c] |
| Aqueous | 11.84 ± 1.1[b] | 16.25 ± 2.0[a] | 17.31 ±1.04[a] | 19.72 ± 2.5[a] | 28.32 ±1.1[a] |
| **Standards** | | | | | |
| Ascorbic acid | 14.50 ± 2.20[a] | 17.35 ± 3.10[a] | 21.85 ± 0.10[a] | 26.80 ± 0.70[a] | 30.30 ± 1.20[a] |
| Gallic acid | 32.00 ± 0.60[c] | 35.57 ± 2.30[b] | 36.75 ±1.80[b] | 38.90 ±1.10[b] | 40.20 ± 0.20[a] |

Data are presented as mean ± SD values of triplicate determinations. [a-d]Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

**Table 5.4**: Percentage NO scavenging activities of extracts from various parts *C.glauca*

| *Extracts* | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|
| | **15** | **30** | **60** | **120** | **240** |
| **Leaves** | | | | | |
| Ethanol | $25.7 \pm 1.7^{c}$ | $31.8 \pm 2.9^{b}$ | $36.46 \pm 2.1^{c}$ | $40.1 \pm 2.0^{c}$ | $51.62 \pm 3.0^{c}$ |
| Aqueous | ND | $2.6 \pm 0.1^{a}$ | $13.27 \pm 0.6^{b}$ | $24.12 \pm 0.1^{b}$ | $37.24 \pm 0.1^{b}$ |
| **Root** | | | | | |
| Ethanol | $29.26 \pm 0.9^{c}$ | $35.87 \pm 1.5^{c}$ | $41.29 \pm 0.8^{c}$ | $63.15 \pm 1.6^{c}$ | $89.33 \pm 0.7^{c}$ |
| Aqueous | ND | $11.73 \pm 0.3^{a}$ | $23.9 \pm 0.5^{b}$ | $28.22 \pm 0.5^{b}$ | $36.91 \pm 0.5^{b}$ |
| **Standards** | | | | | |
| Ascorbic acid | $51.44 \pm 0.05^{c}$ | $56.94 \pm 0.90^{c}$ | $58.37 \pm 1.40^{c}$ | $62.44 \pm 0.70^{c}$ | $77.22 \pm 1.20^{c}$ |
| Gallic acid | $43.06 \pm 9.20^{b}$ | $47.36 \pm 0.70^{c}$ | $49.76 \pm 0.70^{b}$ | $59.33 \pm 3.20^{c}$ | $64.59 \pm 6.20^{c}$ |

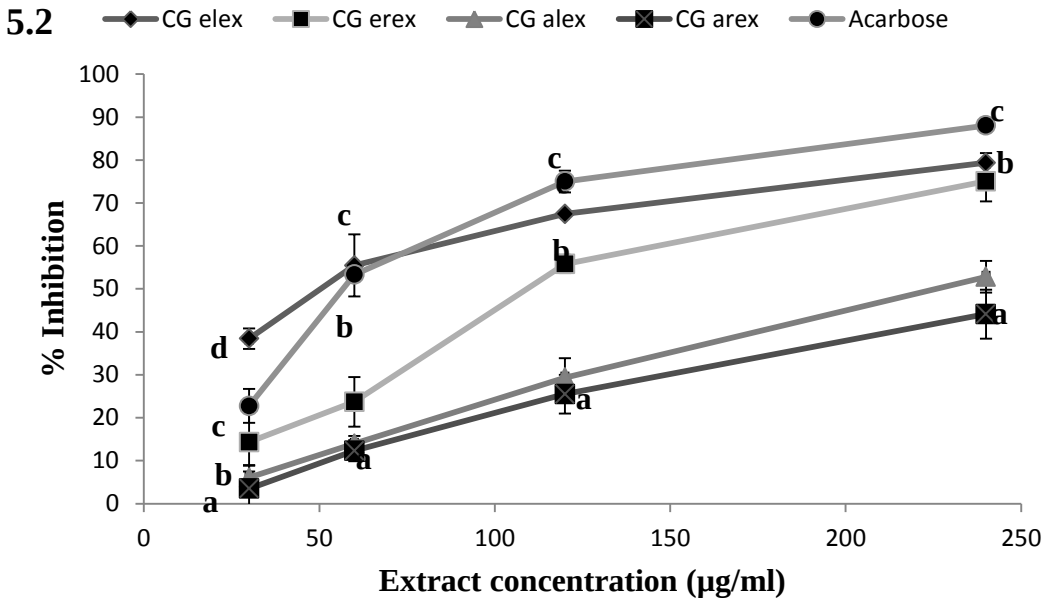
Data are presented as mean ± SD values of triplicate determinations. $^{a-d}$Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$);ND not determined

**Table 5.5:** $IC_{50}$ values of various solvent extracts of *C.glauca* parts in different *in vitro* models

| | IC 50 values in µg/ml | | |
|---|---|---|---|
| Samples | DPPH | HRS | NO |
| **Leaves** | | | |
| Ethanol | 16.26 ±1.23 [b] | 22.53 ± 4.53 [a] | 174.16 ± 23.24 [e] |
| Aqueous | 79.45 ± 6.55 [d] | 285.50 ± 12.76 [d] | 544.51 ± 31.98 [f] |
| **Root** | | | |
| Ethanol | 14.36 ± 2.11 [b] | 21.36 ± 2.78 [a] | 7.69 ± 1.05 [a] |
| Aqueous | 31.18 ± 4.32 [c] | 102.51 ± 8.97 [c] | 123.33 ± 14.17 [d] |
| **Standards** | | | |
| Ascorbic acid | 5.01 ± 0.76 [a] | 64.65 ± 7.78 [b] | 15.13 ± 3.22 [b] |
| Gallic acid | 5.62 ± 2.34[a] | 6.30 ± 205.67 [e]* | 41.68 ± 2.90 [c] |

The * represent the units of these values which are in mg/ml. Data are presented as mean ± SD values of triplicate determinations. [a-f]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

We also investigated the inhibition activity of *C. glauca* ethanol and aqueous extracts on α-amylase and α-glucosidase. Fig. 5.2 and 5.3 shows the inhibitory activities of *C. glauca* extracts on α-amylase and α-glucosidase respectively. In Fig. 5.2, the ethanol leaf extract was found to have a better α-amylase inhibition activity with an $IC_{50}$ of 50.25±1.23µg/mL (Table 5.6). In Fig. 5.3, the ethanol leaf and root extracts were shown to have better α-glucosidase inhibition activities with $IC_{50}$ values of 35.85±5.21 µg/mL and 42.34±1.23 µg/mL respectively (Table 5.6), which were all significantly lower than that of acarbose (standard).



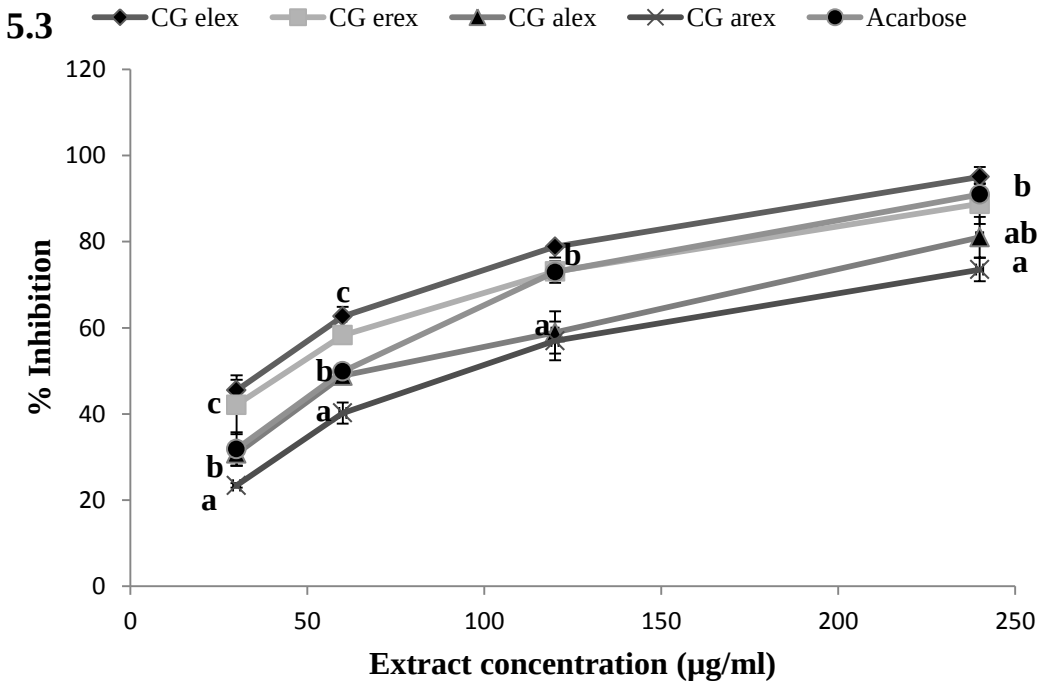




**Fig. 5.2 and 5.3**: **α**-amylase and **α**-glucosidase inhibitory activity of *C.glauca* aqueous and ethanol extracts respectively. **Alex**= aqueous leaf extract; **Elex**= ethanol leaf extract; **Arex**= aqueous root extract; **Erex**= ethanol root extract. Data are presented as mean ± SD values of triplicate determinations. [a-d]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05).

**Table 5.6**: $IC_{50}$ values for α-amylase and α-glucosidase inhibition activity of *C.glauca* extracts.

| | **$IC_{50}$ values in (µg/mL)** | |
|---|---|---|
| **Name of extract** | **α-Amylase** | **α-Glucosidase** |
| **Cg elex** | $50.27 \pm 1.23^{a}$ | $35.82 \pm 5.21^{a}$ |
| **Cg alex** | $252.20 \pm 2.11^{c}$ | $68.68 \pm 3.21^{b}$ |
| **Cg erex** | $109.16 \pm 4.01^{b}$ | $42.34 \pm 1.23^{a}$ |
| **Cg arex** | $368.53 \pm 2.82^{d}$ | $90.18 \pm 4.32^{c}$ |
| **Acarbose** | $52.11 \pm 0.56^{a}$ | $57.18 \pm 3.54^{b}$ |

Data are presented as mean ± SD values of triplicate determinations. **Alex**= aqueous leaf extract; **Elex**= ethanol leaf extract; **Arex**= aqueous root extract; **Erex**= ethanol root extract. $^{a-d}$Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

Fig 5.4 shows the cytotoxicity activity of the *C.glauca* extracts on HEK 293 kidney cell lines as confirmed by the MTT assay. The *C. glauca* ethanol root and leaf and the aqueous root extract did not show any significant decrease in cell viability across all tested concentrations (50 – 200 µg/ml). This is also consistent with the high $IC_{50}$ (Table 5.5) values obtained for *C. glauca* ethanol root and leaf and the aqueous root extracts which are 3.22 mg/ml, 3.52 mg/ml and 822.45 µg/ml respectively. However, *C.glauca* aqueous leaf extract was shown to be toxic because it significantly decreased the viability of cells down to approximately below 50% at 200 µg/ml and also had significantly lower $IC_{50}$ values (Table 5.4) with a value of 170.76 µg/ml compared to the other extracts of the plant.

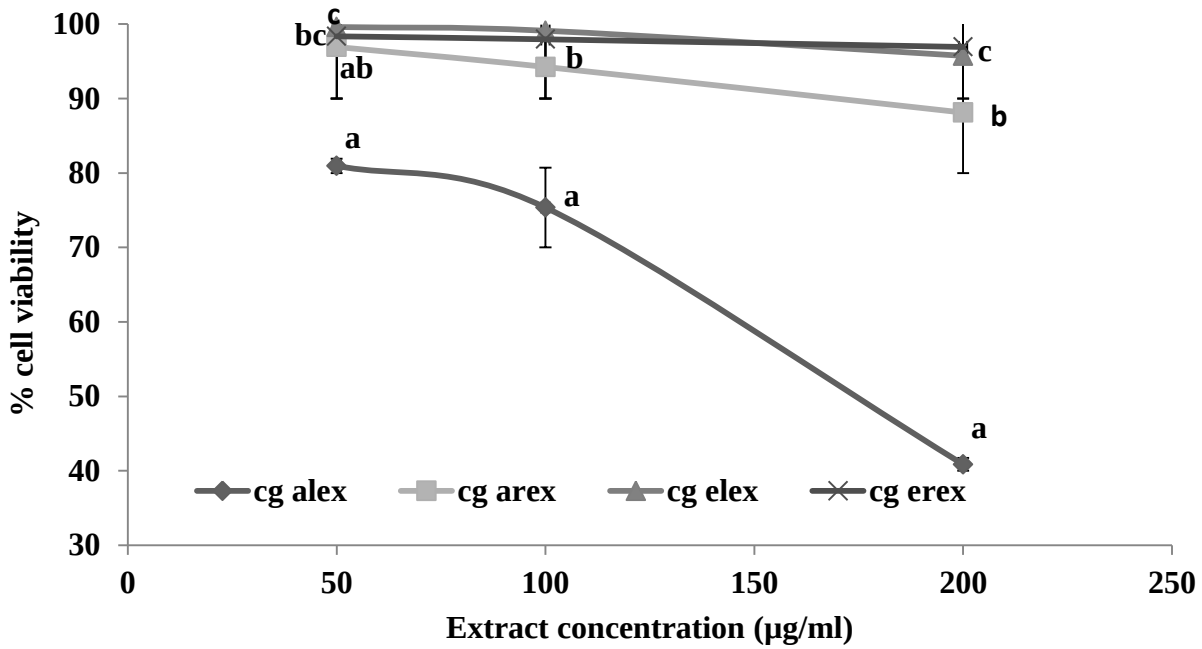



**Fig. 5.4**: Cytotoxicity activity *C.glauca* extracts on HEK 293 kidney cells lines as confirmed by MTT cell proliferation assay. **Alex**= aqueous leaf extract; **Elex**= ethanol leaf extract; **Arex**= aqueous root extract; **Erex**= ethanol root extract. Data are presented as mean ± SD values of triplicate determinations. [a-d]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05).

**Table 5.7**: $IC_{50}$ values for cytotoxicity activity of *C.glauca* extracts on HEK 293 kidney cells.

| **Name of extract** | **Cytotoxicity $IC_{50}$ (µg/mL)** |
|---|---|
| **Cg elex** | $3.52 \pm 0.23^{a\,*}$ |
| **Cg alex** | $170.76 \pm 12.11^{c}$ |
| **Cg erex** | $3.22 \pm 0.14^{b\,*}$ |
| **Cg arex** | $822.45 \pm 22.82^{d}$ |

The * represent the units of these values which are in mg/ml. Data are presented as mean ± SD values of triplicate determinations. [a-d]Different superscripts letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05).

The ethanol and aqueous extracts of the roots and the leaves were subjected to GCMS analysis in order to identify components in the extracts by comparison with mass spectral data of standards

contained in the NIST library. The results indicated the presence of the dihydroxy phenols; resorcinol, catechol and hydroquinone and methoxylated phenols, 2-methoxyphenol and 3,4-dimethoxyphenol as well as an aromatic ester, methyl benzoate, a derivative of benzoic acid, which is a known preservative (Table 5.8; Fig. 5.5).

**Table 5.8:** Compounds identified by GC-MS from the EtOH and aqueous extracts of the leaves and roots of *C.glauca*

| **Name of compound** | **Retention time (mins)** | **Percentage match (%)** | **Molecular mass (g/mol)** |
|---|---|---|---|
| **Ethanol root extract** | | | |
| catechol | 10.56 | 76 | 110 |
| Resorcinol | 11.76 | 89 | 110 |
| Hydroquinone | 11.78 | 81 | 110 |
| **Ethanol leaf extract** | | | |
| 2-methoxyphenol (guaiacol) | 8.21 | 82 | 124 |
| Resorcinol | 14.39 | 72 | 110 |
| **Aqueous root extract** | | | |
| 2-methoxyphenol (guaiacol) | 8.87 | 85 | 124 |
| Methyl benzoate | 8.89 | 87 | 136 |
| Catechol | 10.43 | 91 | 110 |
| 3,4-dimethoxyphenol | 12.56 | 82 | 154 |

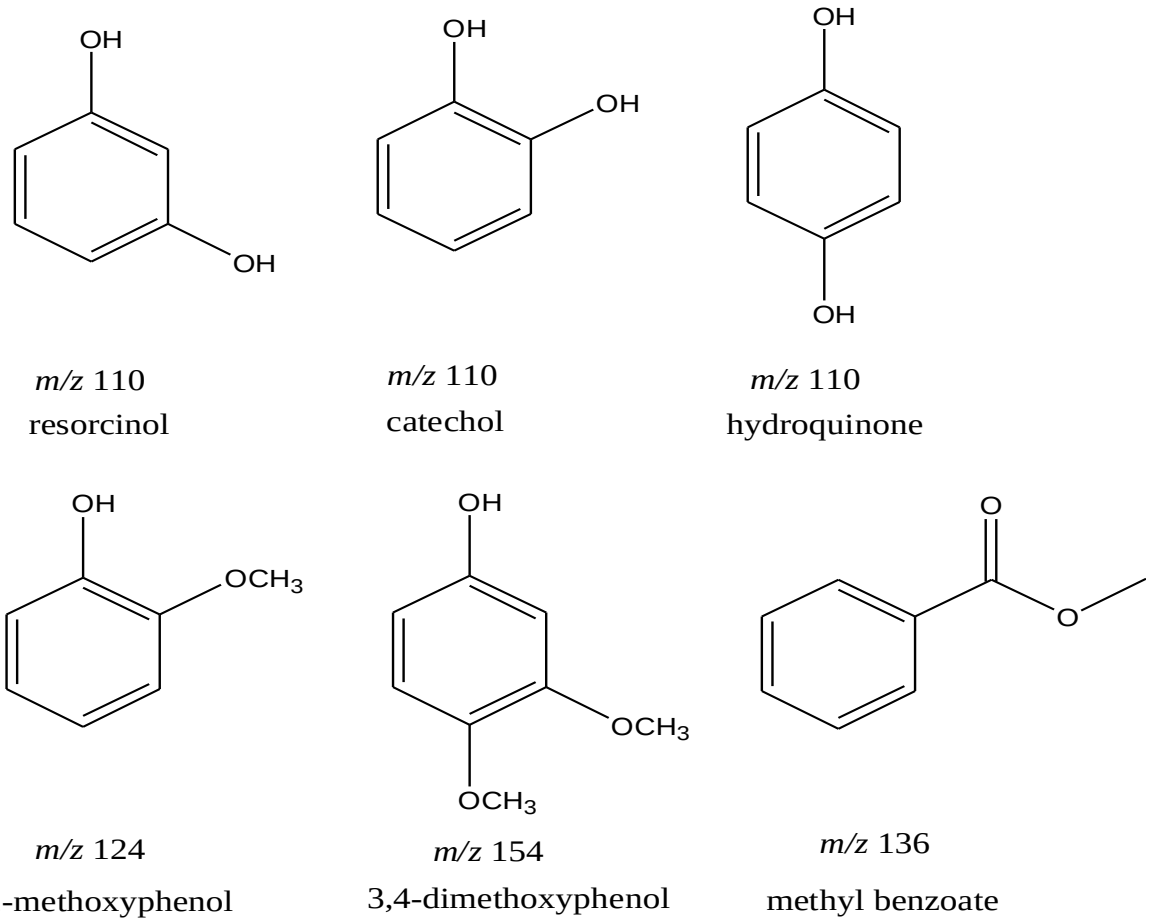



**Fig. 5.5**: Compounds identified by GCMS in the various extracts of *C. glauca* leaves and roots with their mass to charge ratios indicated for each compound as detected by the GCMS instrument.

### 5.1.6 Discussion

*Courbonia glauca* is used by traditional healers to treat and manage non-metabolic chronic disorders in Mrewa district Zimbabwe. In this study, we determined the anti-oxidative as well as α-amylase and α-glucosidase inhibitory activities of the ethanol and aqueous extracts from the leaves and roots in the hope of finding a lead in the search for extracts with potential compounds that could be useful in retarding postprandial hyperglycemia and oxidative stress-associated complications in type 2 diabetes.

The significantly ($P<0.05$) higher antioxidant ability of the ethanol extract of the root indicate that the phytochemical components with high redox potential in these plant parts are more

soluble in ethanol than water. As indicated by Hinnerburg *et al*., (2006), the degree of the free radical reduction is reflective of the radical scavenging (antioxidant) power of the compound. The results of the DPPH assay of the aqueous and ethanol extracts of the leaves and roots revealed that virtually all extracts possessed stronger free radical scavenging potential compared to gallic and ascorbic acids. Notably were the ethanol extracts which indicated to have a significantly high ability to scavenge free radicals at 240 µg/ml which was not significantly ($P<0.05$) different from gallic and ascorbic acids.

As indicated in Tables 5.3 and 5.4, the ethanol extracts of the leaf and root have significantly high NO and hydroxyl radical scavenging ability which was concentration dependent as compared to the aqueous extracts and the two standards (gallic acid and abscorbic acid). Table 5.5 also indicated that the ethanol root extract showed $IC_{50}$ values which were lower than the standards used and hence had better antioxidant potential across all tested *in vitro* models. This observed difference between extracts can be linked to the way in which the nitric oxide and hydroxyl radicals are generated and the different physical and chemical properties of the antioxidants contained in the extracts (Schwarz *et al*., 2001).

The present study showed that the *C. glauca* ethanolic leaf extract moderately inhibits α-amylase and α-glucosidase to a significant extent as evidently shown by the $IC_{50}$ values (Table 5.6). This properly supports an effective T2DM management because it was shown to be a strong inhibitor of intestinal α-glucosidases and a mild inhibitor of pancreatic α-amylase (Krentz and Baile, 2005). To the best of our knowledge, we did not find any prior reports on the antidiabetic (specifically α-amylase and α-glucosidase inhibition) activity of this plant. The ethanolic leaf extracts shows to have both α-amylase and α-glucosidase inhibitory activity however it strongly inhibits α-glucosidase, whereas the ethanolic root extract showed a mild α-glucosidase inhibition activity. α-Amylase and α-glucosidase could potentially be applied as an effective approach in post prandial hyperglycemia management in diabetes type 2 therapy.

Fannell *et al*. (2004) reported that despite widespread use of plants in medicine, they are also potentially toxic at certain concentration ranges and hence some plants are unsuitable for therapeutical applications. A literature search revealed that no prior work was done on the cytotoxicity of *C. glauca* and no toxicity was reported throughout its use in treating a variety of ailments. In this study, the HEK 293 kidney cell lines were used to investigate the cytotoxicity of

*C.glauca* and it was confirmed by using the MTT cell proliferation assay. This technique is based on the ability of succinate dehydrogenase enzymes present in the mitochondria of viable cells to reduce the yellow water soluble substrate MTT into an insoluble, purple formazan product that is further dissolved with the use of DMSO solution. Since this process may occur only in viable, metabolically active cells, the level of activity is the measure of viability. *C. glauca* ethanol root and leaf extracts and the aqueous root extract did not show any significant decrease in cell viability across all tested concentrations (50 – 200 µg/ml). This indicated that the concentration range tested for these plant parts is safe at least in an *in vitro* model and also proved to have significant activities across a set of antioxidant and enzyme inhibition models. However, the aqueous leaf extract displayed cytotoxicity at all tested concentration and hence is not recommended for therapeutical applications at any concentration approximately ≥50 µg/ml.

The identification of the dihydroxyphenols and the methoxyphenols in both ethanol extracts and the aqueous root extracts further support the notion that these extracts have the potential to be used as antioxidants as these compounds are capable of donating a hydrogen atom to a radical species and quenching them. The generated oxygenated radical of the aromatic species has the ability to be delocalized on the molecule which renders them more stable than other radical generated species, hence their ability to act as antioxidants. The ethanol root extract was seen to have three such compounds, resorcinol, catechol and hydroquinone (Fig 5.5) and therefore from a chemical point of view, this extract was seen to have more antioxidant potential than the other extracts. Phenolics have been reported to be potent α-glucosidase and α-amylase inhibitors in some previous and recent studies (de Sousa *et al*., 2004, Hanamura *et al*., 2005, Thilagam *et al*., 2013). So the observed inhibitory activity of the extracts might be caused by the identified phenolics.

This study provided data to support the use of *C. glauca* roots as an antidiabetic agent that could be used by traditional healers in Zimbabwe. All the extracts displayed an impressive ability to inhibit key carbohydrate digesting enzymes. Normally, the aqueous extract is what is used to for medicine; however the ethanol extract has indicated significantly better activity and to be safer than the aqueous extract. Further phytochemical and pharmacological studies will be required to formulate the ethanolic extract of the roots into ethnomedicine.

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# CHAPTER 6

## GENERAL DISCUSSION AND CONCLUSION

### 6.1.1 General Discussion

The global health burden posed by diabetes mellitus is tremendously increased at an alarming rate and ultimately motivated the search for better treatment options. Diabetes mellitus (DM) has turn out to be one of the worst killer disease globally, it's being projected to be the 7th worst killer diseases by 2030 (WHO, 2011). Its high prevalence has also overburdened a lot of economies due to the high costs of treatments of its associated late complications. Also in the light of the fact that the current treatment options have lots of side effects which come along with their prolonged usage due to their synthetic nature. This and other factors have called for a need to explore other options specifically of natural origin, basing on the fact medicinal plants have been in use especially in developing nations for ages and also to a significant extent had influenced the discovery of most modern day therapeutical compounds and also few side effects have been reported from their use.

Hyperglycemia is the hallmark of T2D mellitus (most prevalent form of diabetes mellitus), targeting any of its pathogenic pathways will go a long way in preventing the disease and its late complications. Hyperglycemia has also been the major cause of oxidative stress (OS), the combination of these two conditions has been reported to be major biological players for the pathogenesis of T2D. In this study, we investigated the various antioxidant and anti-hyperglycemic activities of crude extracts of three Zimbabwean plants (*A.antunesiana, C.cornifolia and C.glauca)* using various *in vitro* model and also elucidated their safety.

In our study, we observed that all the antioxidant activities were to a greater extent linked to the total polyphenolic content. The higher the total polyphenol content of an extract, the more potent was its antioxidant power. This can be explained by the nature of polyphenols which have the ability to donate a hydroxyl group or electron that will react and neutralise the free radical, hence termed radical scavengers. In all three plants, crude extracts had much pronounced activity and we significantly noticed in the roots extracted with ethanol which suggests that possibly the most bioactive compound(s) of these Zimbabwe-harvested plants is much prevalent in this part of the plants and also much more soluble in ethanol than water. Based on this observation, we recommend that it is more effective to use ethanol in extracting phytoconstituents from these plants in order to maximize therapeutical benefits of these medicinal plants.

*Albizia antunesiana* recorded highest polyphenolic content also consistently show higher antioxidant power across all models used. A number of benzene related polyphenols; coumarin and triterpenoids were identified in this plant and notably were its highest activity in the ethanol root part (Table 3.6). Apart from the ability to scavenge free radicals, we also investigated the inhibition of carbohydrates digesting enzymes such as α-amylase and α-glucosidase and all the extracts show the ability to inhibit these enzymes to varying extents and notably is the fact that the enzyme inhibitory activities was directly proportional to the total phenol content (Table 3.1; Fig. 3.5) . The identified polyphenols have been reported to be also effective α-glucosidase and α-amylase inhibitors. *Albizia antunesiana* have shown to have better antioxidant potential than the rest of the plants hence a better source of antioxidant compounds and furthermore it did not have a significant cytotoxicity activity.

*Cissus cornifolia* also recorded same trend as *Albizia antunesiana* as an impressive anti-diabetic agent source through free radical scavenging and inhibition of carbohydrates digesting enzymes. The ethanol root extract displayed a consistently high activity across all antioxidant models which are probably due to the high polyphenol content observed (Table 4.1; Table 4.5). We linked the observed activities to the identified compounds which include benzenediol (resorcinol, catechol and hydroquinone), pyrogallol and vanillin a phenolic aldehyde. These compounds are possibly the bioactive components of the extracts hence the higher antioxidant activities observed (Table 4.1; Table 4.5; Table 4.6). The ethanol root extract apart from being the better antidiabetic it also displayed to not cause significant decrease in cell viability hence it safer for therapeutical application at least *in vitro*. *Cissus cornifolia* extracts overally show to be intermediate antidiabetic agent source.

*Courbonia glauca* antidiabetic potential also followed the same trend with *A.antunesiana* and *C.cornifolia*. The ethanol extracts were better antioxidants and anti-hyperglycemic agents than aqueous extracts and this was due the higher polyphenols extract in ethanol extracts. These compounds are well known to be good radical scavengers and also α-glucosidase and α-amylase inhibitor (de Sousa *et al*., 2004, Hanamura *et al*., 2005, Thilagam *et al*., 2013). The ethanol extracts displayed the least cytotoxicity activity on Human Embryonic Kidney cell lines (HEK 293) as compared to aqueous extracts (Fig. 4.6; Table 4.7). However despite all the anti-diabetic activity by extract of *Courbonia glauca,* they were the least active extracts of the three tested

plants and this is also confirmed with lowest total polyphenol content of the three (*A.antunesiana, C.cornifolia and C.glauca).* This further confirms our earlier assertions that most of the biological activities measured, at least for the plants under study are mediated by polyphenolics. Hence, phenolics from these plants could serve as antidiabetic agents.

The observed trends in the data presented for the three plants can further be substantiated by the nature of phytoconstituents observed in the extracts of these plants by GC-MS. The antioxidant activities recorded might have been mediated at least in part through the donation of $OH^-$ group and accepting of electrons which will result in neutralization of the resultant radical(s). In *A.antunesiana* we detected isomers of benzene diol, benzene triol, coumarins double ringed, and multiple benzene ringed tripernoids such as amyrin. This indicates the presence of compounds with a lot of free hydroxyl groups hence better capacity to quench radical(s). In extracts of *C.cornifolia* we detected isomers of benzene diol, benzene triol and vanillin (phenol aldehyde), which also have a moderate ability to quench radicals however not as much as *A.antunesianna* extracts. In *C. glauca* extracts we detected isomers of benzene diol and a variety phenolic compounds conjugated to other group e.g. methyl and mainly there were only having single $OH^-$ group available hence the least radical scavenging ability of the three. Since the enzyme inhibition ability has been reported to be strongly linked to the phenolic content the plant (Table 5.1; Fig. 5.2; Fig. 5.3; Table 5.6). Hence better extracts in terms of antioxidant and anti-hyperglycemic power were from *A.antunesianna* followed by *C.cornifolia* and the least were from *C. glauca*, regardless of these difference some extracts from these plants specifically the ethanol root parts showed encouraging results which warrants further investigations. Aqueous extracts were the more toxic of the two solvents used hence not encouraged for therapeutic applications.

Our data show that all extracts from different plants we investigated followed a general trend wereby the aqueous extracts caused much decrease in cell viability than ethanol extracts. Also the ethanol root extracts did not caused much cell death as observed in leaf extracts and overally the *Albizia antunesiana* cause the least cell death. Looking at the observed trends it clearly indicates that the bioactive components were mostly extracted from the roots part and the least activity and much cytotoxicity was witnessed with leaf extracts. The given data also supports the use of the root part of the plants for medicinal purposes which might be all based on the high

polyphenolic content of these parts among other bio-components. However, the observed trends are impressive enough to take the extracts which displayed high activities for further analysis *in vivo*.

However, results obtained *in vitro* might tend to differ from *in vivo* assays since there are a lot of factors that plays various and significant interfering roles under *in vivo* conditions. Hence, in order to completely conclude on the bioactivity of compounds both *in vitro* and *in vivo* results need to agree. Thus, further investigation need to be done using *in vivo* models in order to verify if the observed activities will be maintained inside a living system

### 6.1.2 Conclusion

Our results on the three plant species (*A.antunesiana, C.cornifolia and C.glauca)* used in the traditional medicine of Mrewa showed that all three species possess remarkable anti-diabetic activity which is mediated, at least in part, through free radical scavenging and α-amylase and α-glucosidase inhibition mechanisms of action. In particular, *A.antunesiana* ethanol root extract showed the highest activity compared to that of other extracts. We strongly believe the high antioxidant capacity observed to be related, to the polyphenolics found in this extract. Our data strongly support the use of the three medicinal plants to treat diabetes as they have displayed to have significant antioxidant and anti-hyperglycemic activities.

### 6.1.3 Recommendations

Further investigation on these plant parts for *in vivo* antidiabetic activity is highly encouraging. We recommend more investigations using partitioned fractions of these plants extracts in order to identify the most active compound(s). Furthermore, the use other specific isolation techniques is encouraged so as to isolate the probable active compound(s) and individually characterize them and if possible develop a functional therapeutic formulation.

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## APPENDIX

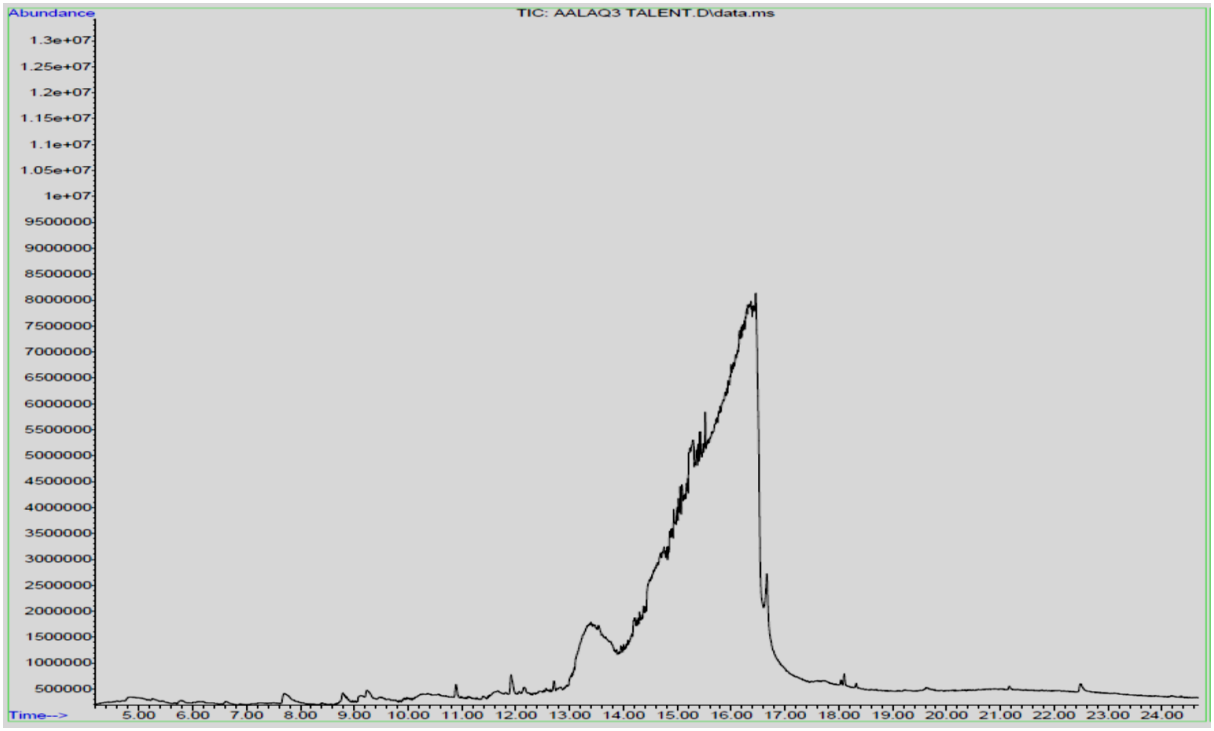


**Fig. AA01:** GC-MS chromatogram of *A.antunesiana* aqueous leaf extract

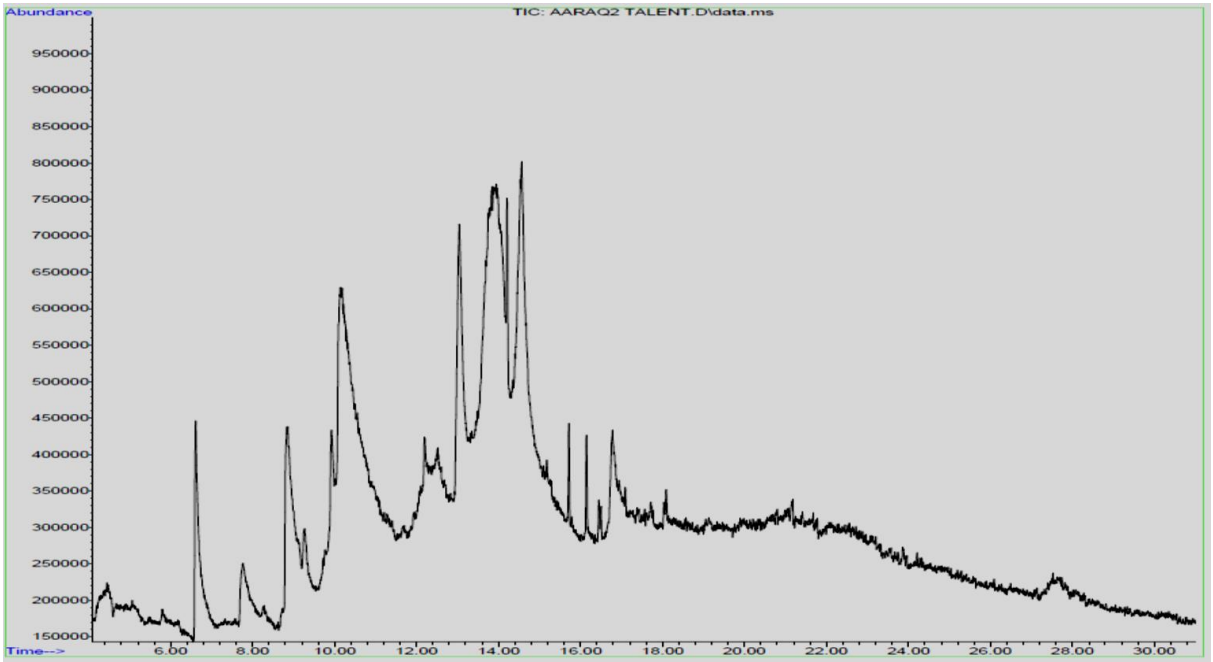


**Fig. AA02**: GC-MS chromatogram of *A.antunesiana* aqueous root extract.

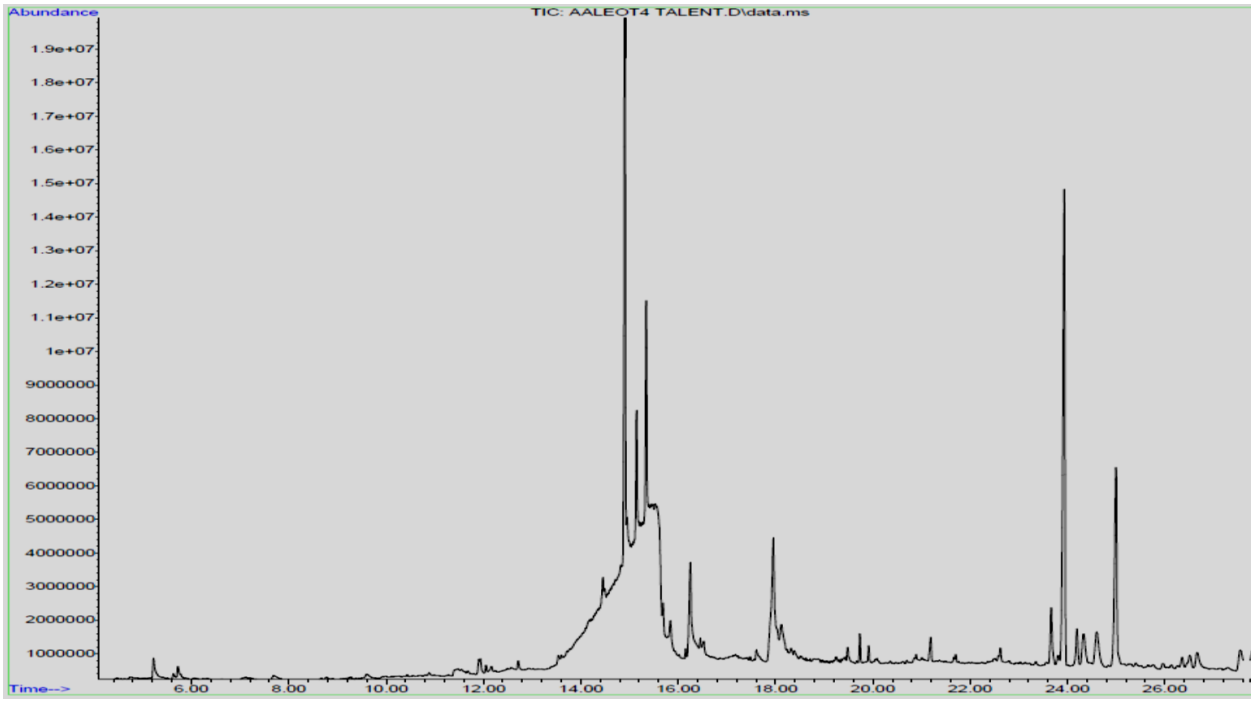


**Fig. AA03** GC-MS chromatogram of *A.antunesiana* ethanol leaf extract**.**

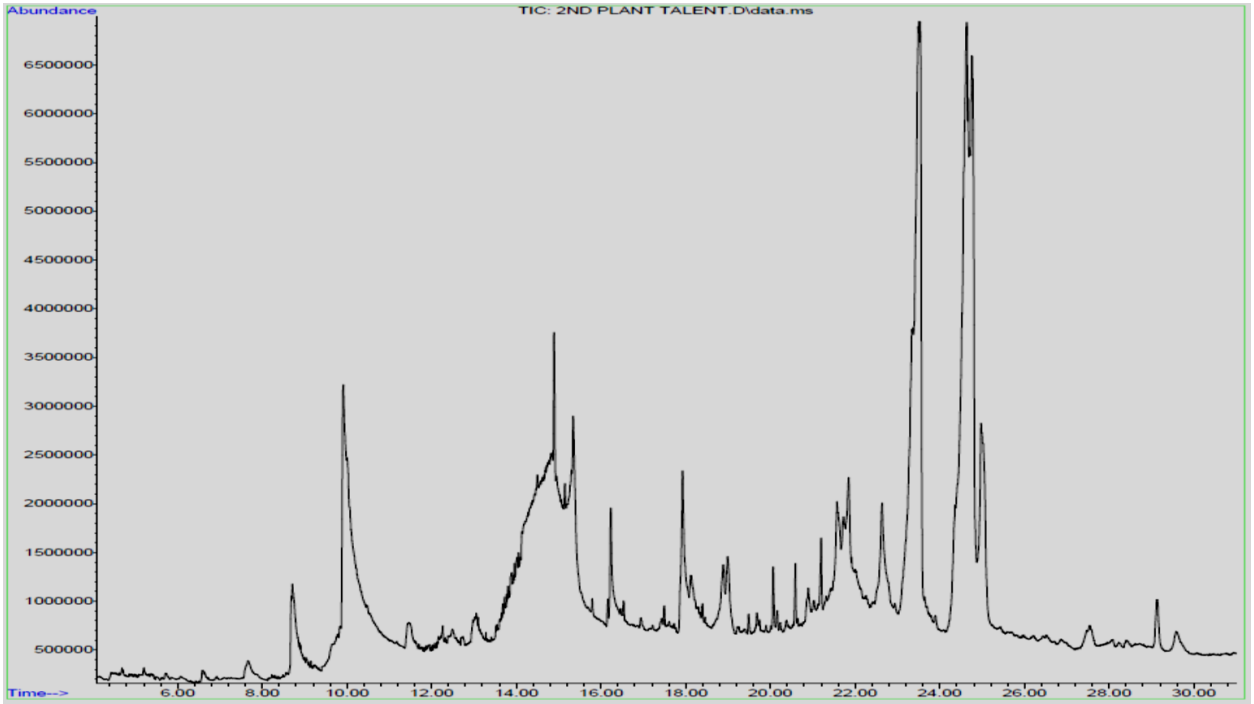


**Fig. AA04:** GC-MS chromatogram of *A.antunesiana* ethanol root extract**.**

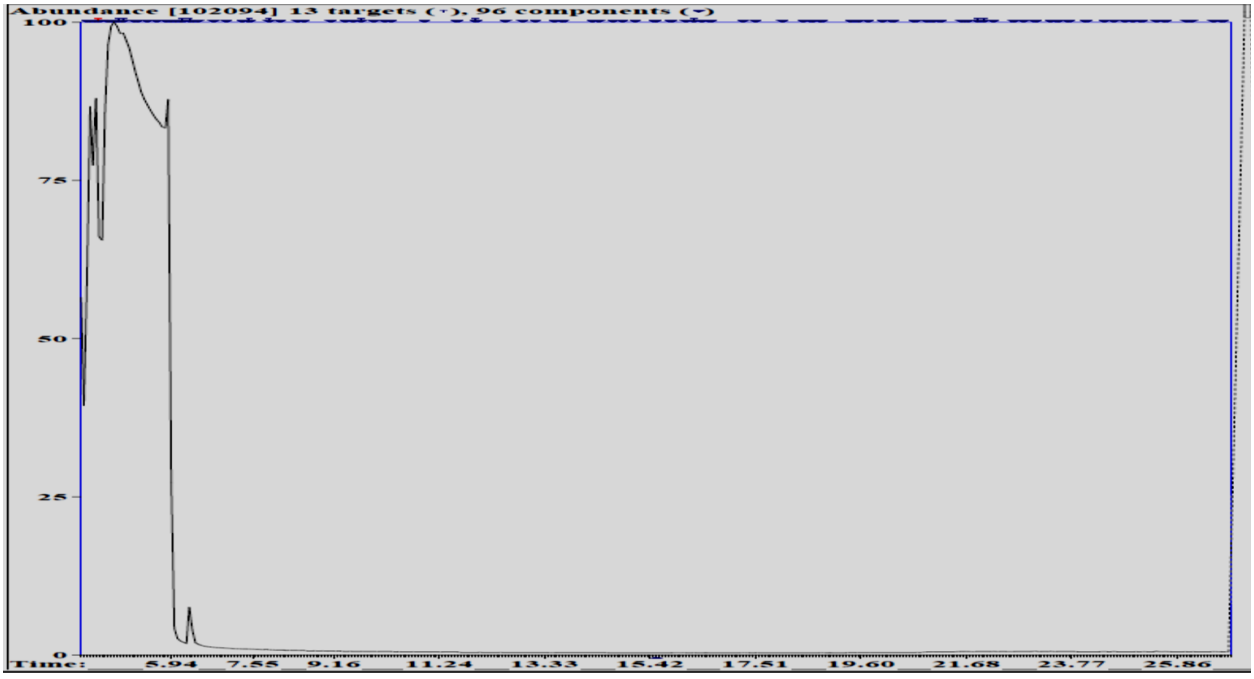


**Fig. CC01:** GC-MS chromatogram of *C.cornifolia* aqueous leaf extract.

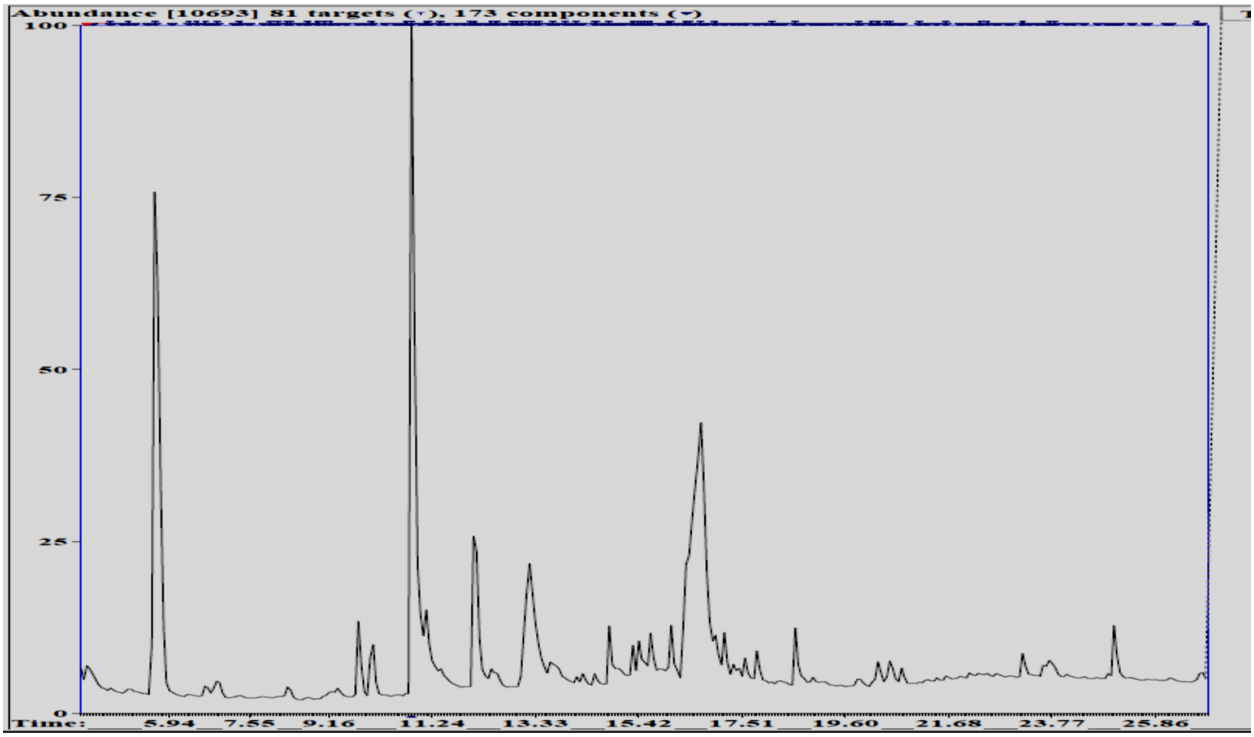


**Fig. CC02**: GC-MS chromatogram of *C.cornifolia* aqueous root extract.

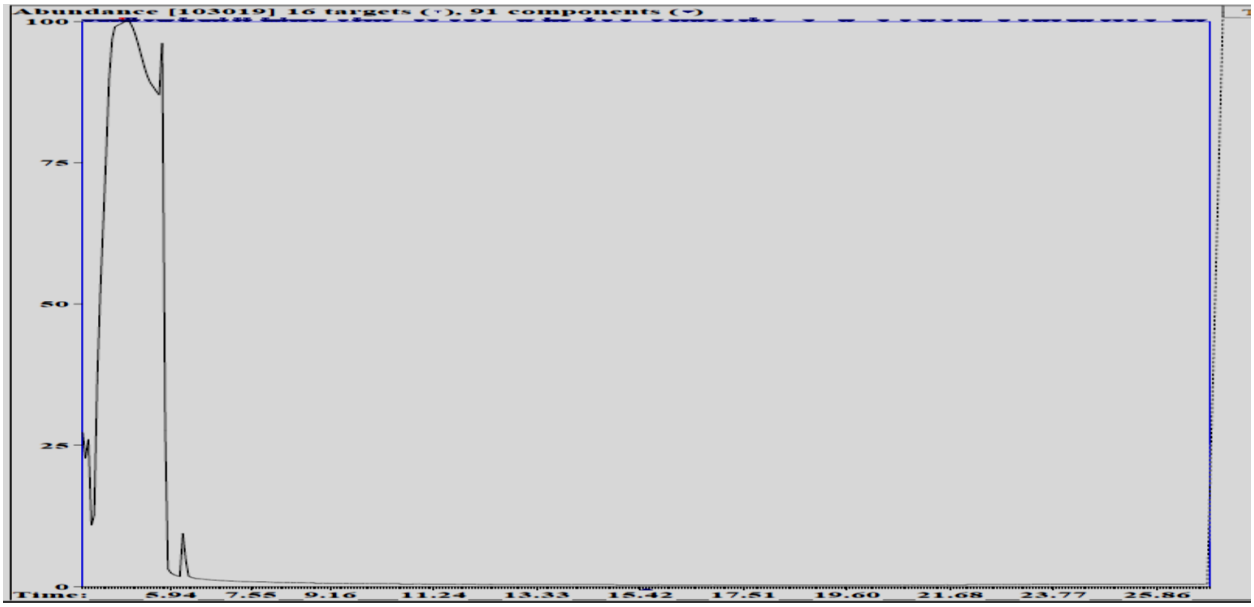


**Fig. CC03:** GC-MS chromatogram of *C.cornifolia* ethanol leaf extract.

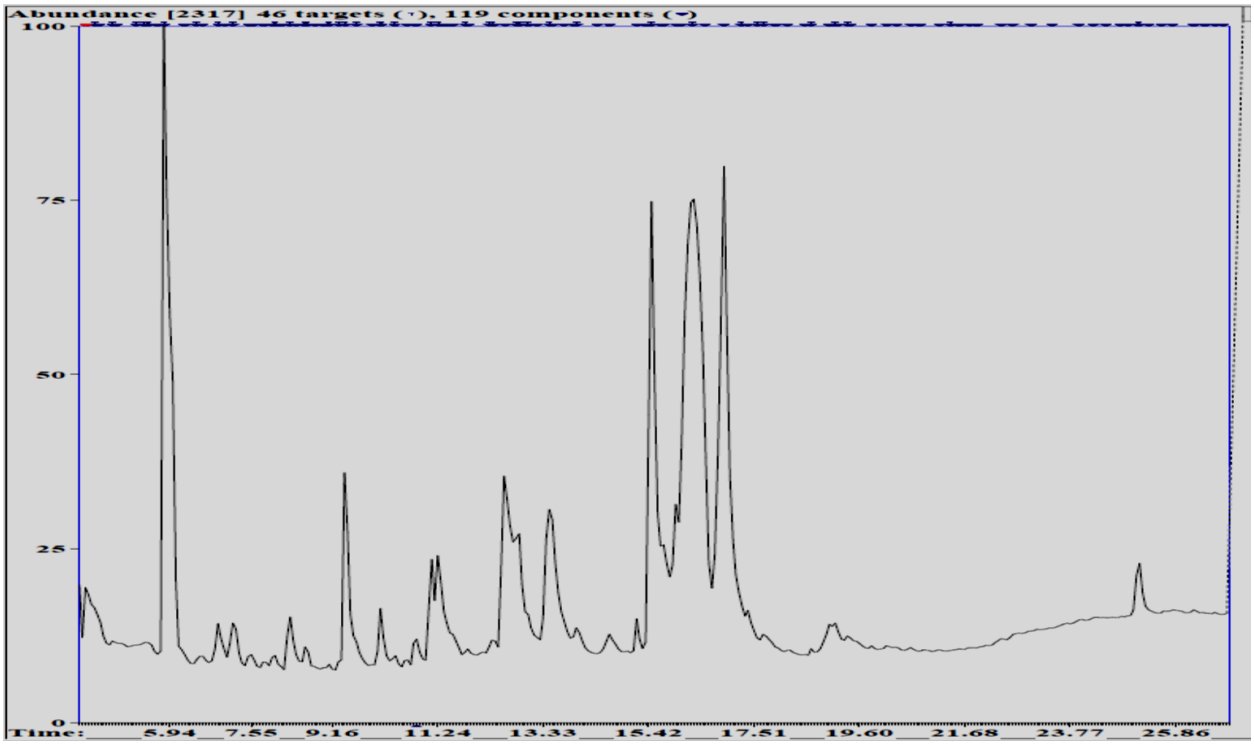


**Fig. CC04**: GC-MS chromatogram of *C.cornifolia* ethanol root extract.

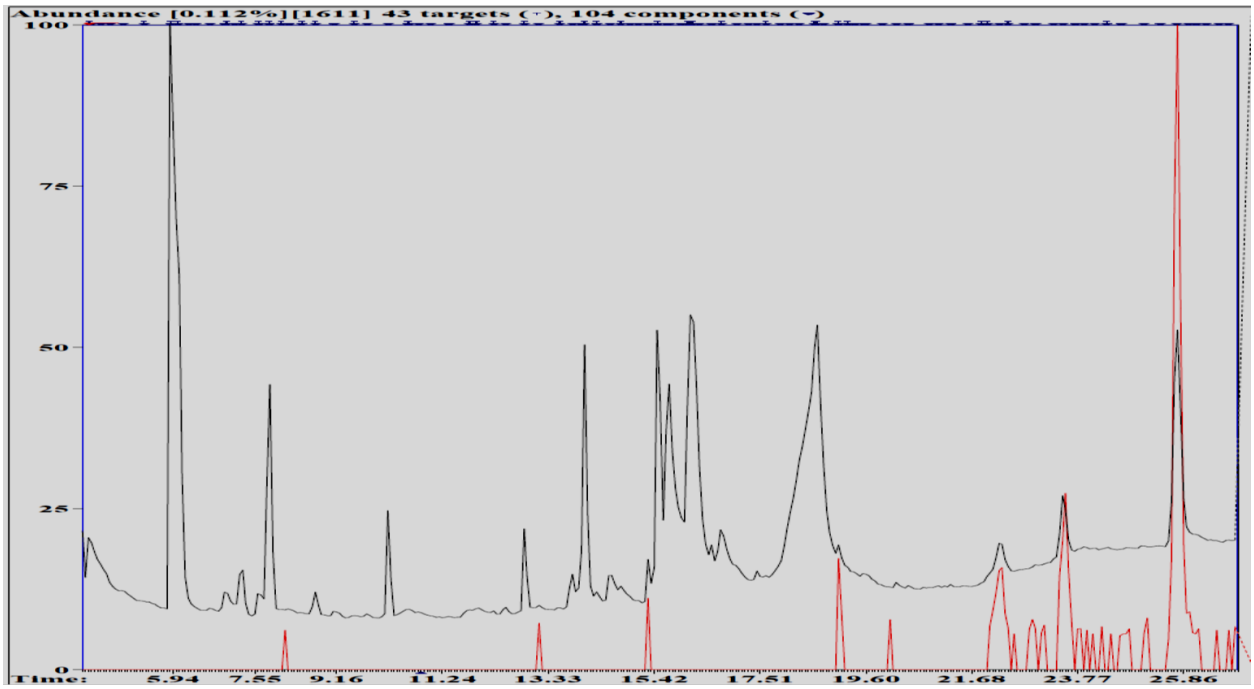


**Fig. CG01:** GC-MS chromatogram of *C.glauca* aqueous root extract

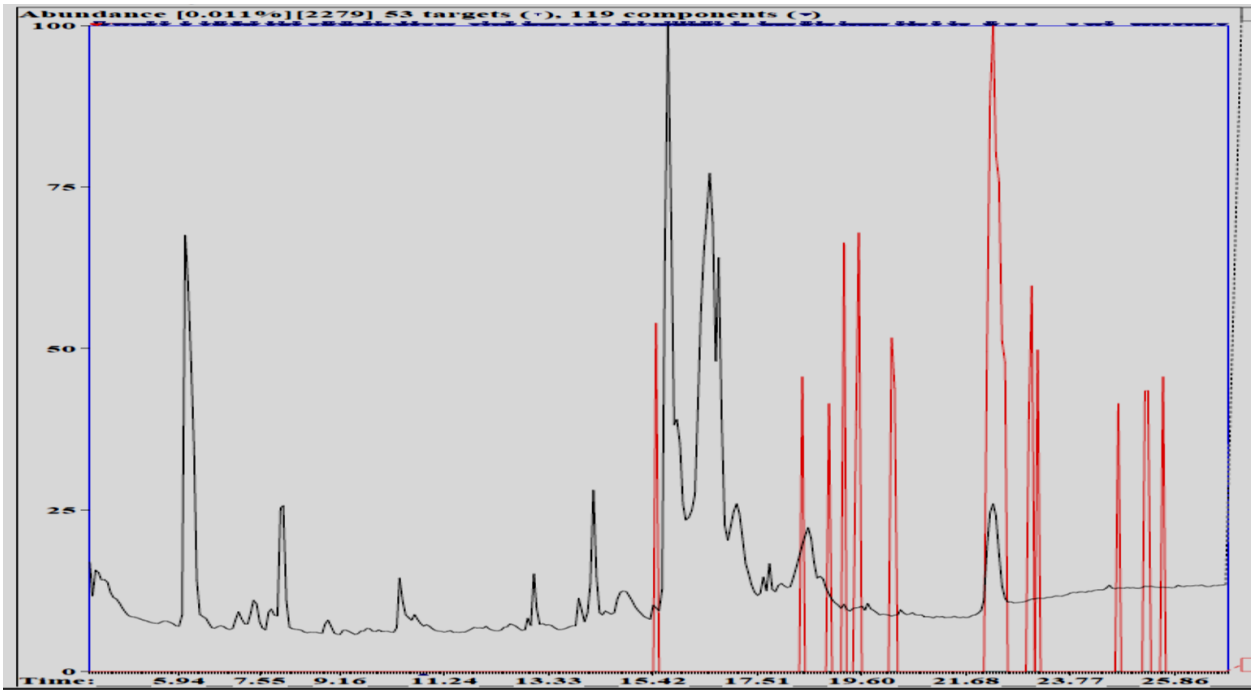


**Fig. CG02**: GC-MS chromatogram of *C.glauca* ethanol root extract