Cape Peninsula
University of Technology

The biochemical effects of *Hypoxis hemerocallidea* in the kidney and liver of streptozotocin-induced diabetic male Wistar rats.

By

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Thesis submitted in fulfilment of the requirements for the

Master of Technology Degree: Biomedical Technology

In the Faculty of Health and Wellness Sciences

At the

Cape Peninsula University of Technology

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Bellville
September 2015

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DECLARATION

I, Mediline Goboza, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed	**Date**

ABSTRACT

Diabetes mellitus (DM) is an endocrine disorder that is characterised not only by severe hyperglycemia but also altered metabolism of glucose and lipids. It is a major health problem worldwide and its impact is greatly noticed in developing countries due to the lack of adequate medical facilities. Oxidative stress remains the principal factor that actively plays major roles in the onset and progression of diabetes mellitus and its complications. The use of medicinal plants in the treatment of DM has undisputedly gained the attention and interest of researchers throughout the globe mainly because plants have established promising outcomes in the treatment of diabetes. It is evident that the plants' constituents possess therapeutically potent metabolites that have beneficial effects such as antioxidant, antidiabetic, anticancer, anti-inflammatory and antibacterial activities. *Hypoxis hemerocallidea* is a native plant that grows in the Southern African regions. *H. hemerocallidea* is well known for its beneficial medicinal values. In South Africa it is known as the African potato. The main aim of this study was to investigate both the beneficial and also the possible toxic effects of *H. hemerocallidea* in the kidney and liver tissues of streptozotocin-induced diabetic male Wistar rats by assessing the antioxidant status and selected biochemical parameters in the two studied organs.

Diabetes was induced in overnight fasted rats by administration of a single intraperitoneal injection of STZ at a dosage of 50mg/kg in citrate buffer (0.1 M at 4.5 pH). Hyperglycemia was confirmed 72 hours after induction of diabetes using STZ in rats with glucose levels > 15 mmol/l. Treatment with the plants extract commenced on the fourth day after STZ administration via gastric gavage that was done once a day over a 6 week period. The effects of *H. hemerocallidea* on glucose, body weight, liver and kidney weights, liver function, kidney function and the oxidative status were evaluated after the feeding period.

The 800 mg/kg and the 200mg/kg dosages of *H. hemerocallidea* lowered the blood glucose levels in STZ-induced diabetic groups. No significant change in blood glucose levels was seen in the normal treated group. Kidney hypertrophy which confirmed kidney damage was observed only in the the diabetic untreated group and also in the relative kidney weights of diabetic treated groups. There was significant increase in relative liver weights in all groups when compared to the normal control.

The kidney function was evaluated by measuring the serum levels of creatinine and albumin. Decrease in serum albumin was observed in all STZ-induced diabetic groups. Treatment with the plant extract did not increase serum albumin level. High levels of creatinine were

observed in the diabetic control group. There were no significant changes in the serum creatinine levels of the entire treated groups when compared to the normal control.

Elevation of the liver function enzymes, a major marker of hepatic injury was observed in the diabetic and the 800mg/kg treated diabetic groups (with increased AST and ALP) with no significant differences between the two groups. The lower dose (200mg/kg) of *H. hemerocallidea* ameliorated the hepatic enzymes levels to near normal but shows no lowering effect on ALP when compared to the normal control group.

Total antioxidant assessment in both tissues was carried out using the FRAP and the ORAC assays. Hyperglycemia reduced the oxygen radical absorbance capacity in the diabetic group. In the liver tissue, both doses significantly increased the oxygen radical absorbance capacity in diabetic treated groups when compared to the diabetic control. On the contrary, the kidney ORAC values in the 800mg/kg treated diabetic group and the diabetic group were lower than in both the normal untreated and the normal treated groups. The plant extracts elevated the liver FRAP in all the treated groups. FRAP activity was however increased in the kidney tissue of the diabetic control and the 200mg/kg treated group.

H. hemerocallidea enhanced the hepatic catalase activities in all the treated groups when compared to the diabetic control, whereas in the kidney tissue, the catalase activity was increased in the diabetic control due to the compensatory response mechanism. Alterations in the SOD and GSHt were observed only in the kidney tissue of normal treated group where there was increase in activities of both enzymes. *H. hemerocallidea* treatment did not alter the activities of liver SOD in all groups when compared to the normal control group. In the diabetic control group, there was significantly decreased liver GSHt activity when compared to the normal and group 5 treated normal rats.

In conclusion; both doses of *H. hemerocallidea* demonstrated anti-hyperglycemic and antioxidant effects especially in the liver tissues whereas the effects of (800mg/kg) in the kidneys did not differ significantly when compared to the diabetic control group.

ACKNOWLEDGMENTS

Firstly, I would like to thank the Lord my God for granting me the opportunity to partake and successfully complete my Masters degree against all odds.

I sincerely thank my supervisor Professor O.O. Oguntibeju and my two co-supervisors Dr S. Meyer and Dr Y.G. Aboua for mentoring, guiding and encouraging me throughout the course of my study, may God bless you.

I extend my sincere gratitude to Mr Fanie Rautenbach, Mrs Olivia Parbhunath and Professor J. Marnenwick for their technical assistance and inputs.

My appreciation also goes to my parents Mr and Mrs N.C. Goboza for being pillars of strength in my life; I wouldn't be where I am today if it was not because of your support. I wish to thank my wonderful siblings Diana, Phumzani, Tongai, Mollyne and my grandfather for your unwavering support, may the Mighty God continue to bless you, I love you all.

I would like to thank my friends, Phumuzile, Mandisa, Emmanuel, Boitumelo, Loice, Audrey, Dr Kayode Olubgenga, Ife Elizabeth, and Bukola for your love and support.

Finally, I sincerely thank the Cape Peninsula University of Technology for the funds that were directed towards this project.

DEDICATION

This thesis is dedicated to my late friend Hilory Munhuweyi

Table of Contents

LIST OF FIGURES

CHAPTER 2

LIST OF TABLES

CHAPTER 2

GLOSSARY

Abbreviations	Definition/Explanation
$O_2^{\cdot-}$	Superoxide radical
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous ion
$ONOO^-$	Peroxynitrite
$NO^{\cdot-}$	Nitrious oxide radical
$HO^{\cdot}$	Hydroxide radical

A

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
AGE	Advanced glycation end products

B

BMI	Body mass index

C

CAT	Catalase
CMV	Cytomegalovirus
CuSOD	Copper-superoxide dismutase
CVD	Cardiovascular diseases

D

DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DAG	Diacylgycerol
DKD	Diabetic kidney disease

E

ETC　　　　　　　　　　　　　　　　　Electron transport chain

ESRD　　　　　　　　　　　　　　　　End stage renal disease

G

GMO　　　　　　　　　　　　　　　　Genetically modified offsprings

GR　　　　　　　　　　　　　　　　　Glutathione reductase

GSH　　　　　　　　　　　　　　　　Reduced Glutathione

GAPDH　　　　　　　　　　　　　　　Glyceraldehyde3- phosphodehydrogenase

GFR　　　　　　　　　　　　　　　　Glomerular filtration rate

GL　　　　　　　　　　　　　　　　　Glycemic load

GLUT　　　　　　　　　　　　　　　　Glucose transporter

I

IRS　　　　　　　　　　　　　　　　Insulin receptor substrates

IDDM　　　　　　　　　　　　　　　　Insulin dependent diabetes mellitus

IR　　　　　　　　　　　　　　　　　Insulin resistance

IFG　　　　　　　　　　　　　　　　Impaired fasting glucose

IGT　　　　　　　　　　　　　　　　Impaired glucose tolerance

L

LDL　　　　　　　　　　　　　　　　Low density lipoproteins

N

NCD　　　　　　　　　　　　　　　　Non-communicable diseases

NIDDM　　　　　　　　　　　　　　　Non-insulin dependent diabetes mellitus

NAFLD　　　　　　　　　　　　　　　Non-alcoholic fatty liver disease

O

OGTT　　　　　　　　　　　　　　　Oral glucose tolerance test

OS　　　　　　　　　　　　　　　　　Oxidative stress

OAD　　　　　　　　　　　　　　　　Oral antdiabetic drugs

P

PKC　　　　　　　　　　　　　　　　Protein kinase C

PBS　　　　　　　　　　　　　　　　Phoshate buffered saline

R

ROS   Reactive oxygen species

RNS   Reactive nitrogen species

RAGE   Receptor for advanced glycation end products

S

STZ   Streptozotocin

SOD   Superoxide dismutase

SDH   Sorbitol dehydrogenase

SRC   Standard rat chow

T

T2DM   Type 2 diabetes mellitus

U

UAE   Urine albumin excretion

V

VEGF   Vascular endothelial growth factor

W

WHO   World health organisation

Chapter 1

INTRODUCTION

1.1 Statement of research problem

Diabetes mellitus (DM) is becoming one of the leading causes of death worldwide because of its adverse complications that include cardiovascular related diseases and chronic kidney disease (Gupta *et al.*, 2003); Islam, 2011). DM has become a menace to public health because of the unavailability of adequate drugs to manage this condition. No patient in medical history has been totally cured of diabetes, despite the use of various drugs and hypoglycemic agents to control and reduce the complications of diabetes (Afolayan and Sunmonu, 2010). Proper management and treatment of diabetes mellitus is lacking, especially in developing countries, which possibly explains the escalating percentages of morbidity and mortality (Dailey, 2011; Afkarin *et al.*, 2013). In Africa, as a result of poor socio-economic conditions, it is nearly impossible to properly monitor and maintain DM. Amod *et al* (2012) stipulated that the prevalence of diabetes differs with ethnicity in South Africa and estimated the prevalence to be 6.2%, 17.1%, 6.4%, and 6.2% in the Coloured, Indian, Black and White communities respectively.

Recent evidence suggested that theincrease of the prevalence of type 2 DM in South Africa is related mostly to the standard of living and the life styles that closely mimic those practised in Western countries (Groenewald *et al.*, 2009). Obesity and poor dietary habits have been linked to the acceleration of the pathogenesis of type 2 DM (Sicree and Shaw, 2007; Erasmus *et al.*, 2012). A marked increase in the consumption of genetically modified offspring (GMOs), animal products as well as fatty foods has become more pronounced in South Africa. In addition to the unfavourable dietary shift, poor body weight maintenance is also a major factor in the development of NCDs (Whiting *et al.*, 2011. In 2008, it was indicated that 87% of the type 2 DM patients in South Africa are obese or overweight (Steyn and Labadarios, 2008). Obesity therefore reinforces the onset of DM (Waugh *et al.*, 2007). Recent epidemiological studies revealed that the obesity trend has shifted from urban societies and is thus affecting poorer and the rural population, the rising trends has been contributed mainly by the country's economic growth (Hu, 2011).
 From this information, it can be hypothesised that DM incidence and prevalence rates are increasing in the rural areas of South Africa.

1.2 Research aim and objectives

The overall aim of this study was to evaluate the biochemical effects of *Hypoxis hemerocallidea* on oxidative stress markers, liver enzymes, as well as other biochemical parameters on the liver and kidney tissues of both normal and STZ-induced diabetic male Wistar rats.

1.3 Objectives

The current study was designed to evaluate the effects of *Hypoxis hemerocallidea* in diabetic and non-diabetic rats. The objectives of the study are indicated below:

1. Measure antioxidant enzymes such as total glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in the liver and kidney tissues.
2. To measure the levels of serum glucose, albumin and total protein.
3. To measure the activities of liver enzymes such as alanine amino transaminase (ALT), aspartate amino transaminase (AST), and alkaline phosphatase (ALP).
4. To determine the oxygen radical absorbance capacity in liver and kidney tissues.
5. To measure the ferric reducing antioxidant power of *Hypoxis hemerocallidea* in fed groups.

Chapter 2
LITERATURE REVIEW

2.1 General overview of diabetes mellitus

Diabetes mellitus (DM) is a term that means passing sweet water and was first discovered by a Greek physician named Arateus in the 2nd century (Zisser *et al.*, 2010). Diabetes mellitus is a complex endocrine disease characterised by chronic hyperglycemia and an attenuated metabolism of lipids, carbohydrates and proteins associated with absolute or relative insulin deficiency in insulin secretion or action (Ghlissi *et al.*, 2013). This defect in fuel metabolism is marked by polyuria, polydipsia, and polyphagia (American Diabetes Association, 2008). Insulin is the principal anabolic peptide hormone secreted by the β cells of the pancreas that plays a fundamental role in the regulation and maintenance of normal glucose and lipid levels (Henriksen *et al.*, 2011). The biosynthesis of insulin involves three main steps:

1. The synthesis of preinsulin on the ribosomes of the rough endoplasmic reticulum.
2. The conversion of preinsulin into proinsulin which is then transported to the secretory granules of the Golgi apparatus.
3. Cleavage of proinsulin into insulin and C-peptide.

2.1.1 Signal Transduction through Insulin

Insulin is released postprandially by the β cells of the Islets of Langerhans found in the pancreas, in response to enhanced levels of glucose in circulation. Insulin performs its paracrine action on several tissues but mainly on skeletal, adipose and the hepatic tissues (Sesti, 2006). These tissues possess a heterotetrameric transmembranal tyrosine kinase receptor composed of two α extracellular binding chains and two intracellular β chains. In response to increased glucose levels in circulation, insulin acts as a ligand, binding to the extracellular α-chains of its receptor. Binding of insulin to its receptor elicits autophoshorylation of the tyrosine sites (Tyr^{960}) located on the intracellular domain of the receptor (Sesti *et al.*, 2001; Bjornholm and Zlerath, 2005). The systemic phosphorylation of the Tyr^{960} results in the formation of a recognition motif for the phosphorylation binding domain of the insulin receptor substrates (IRS). The IRS-1 protein is responsible for the transmission of signals that activates specific biological responses, e.g. glucose

transport and synthesis of macromolecules (Cheng *et al.*, 2002). IRS-1 binds to several proteins with (Src homology 2) SH-2 like p85. P85 is a regulatory subunit of phosphatidylinositol 3- kinase (P13- kinase). After IRS-1 binds to p85, a catalytic molecule P110 is recruited causing a further activation of the 3-phosphoinositide-dependent kinases which as well phosphorylates Akt (a serine/threonine kinase) on Thr[308], the p13 kinase pathway coordinates the translocation process of GLUT 4-a glucose transporter molecule (Bevan, 2001; Sesti, 2006; Henriksen *et al.*, 2011). Glut proteins migrate from the intracellular environment to the cell surface membrane, where their vesicles fuse with the cell membrane, allowing a channel for glucose entry (Figure 2.1) and consequently lowering the glucose levels in circulation (Byon *et al.*, 1998; Storgaard *et al.,* 2001).
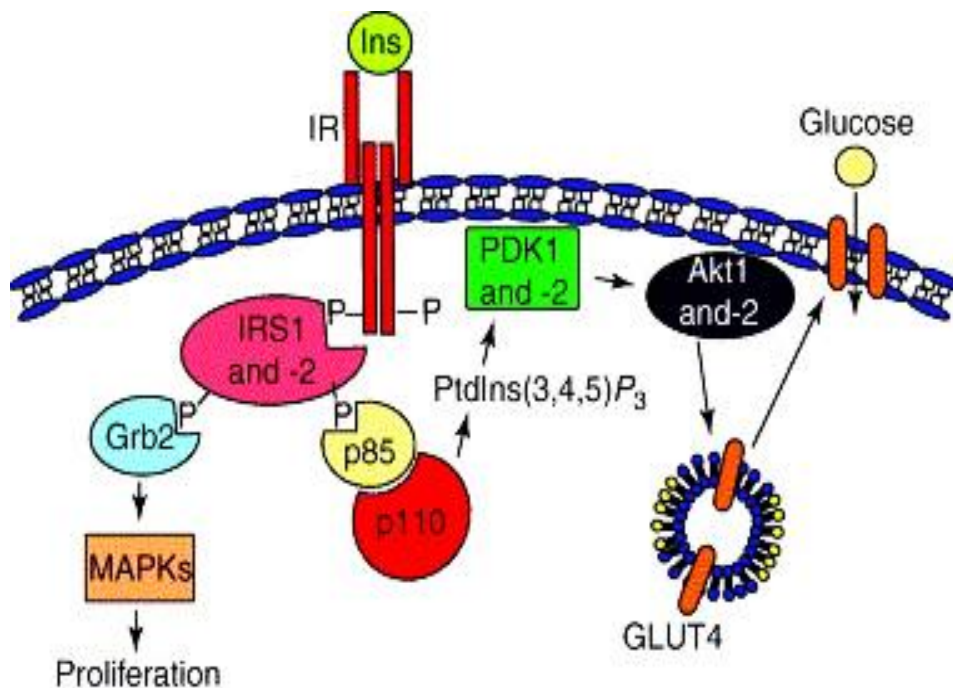
Figure 2.1: Insulin signalling cascade (Thirone *et al.,* 2006)

Abbreviations: IR: insulin receptor, Ins: insulin, IRS1: insulin receptor 1, ptdlns(3,4,5)P_3: PI3, GLUT4: glucose transporter type 4 molecule, PI3 kinase pathway include 3 phosphoinositide-dependent kinase 1 and-2 (PDK 1 and 2) which phosphorylates Akt 1 and 2 resulting in translocation of GLUT4 into the plasma membrane

Other effects of normal insulin signalling include: Increased rate of glycogenesis, inhibition of glucagon secretion by alpha cells, increased lipid and protein storage and synthesis, inhibition of gluconeogenesis and glycogenolysis, increased rate of glycolysis (Aronoff *et al.,* 2004).

2.2 Classification of Diabetes Mellitus

As mentioned in the above text, diabetic patients cannot produce or properly use insulin i.e. defective insulin metabolism. Depending on the aetiology, several types and classifications of diabetes exist where all types exhibit either defective insulin metabolism or production.

2.2.1 Type 1 Diabetes Mellitus (DM)

Type 1 diabetes mellitus also referred to as insulin dependent diabetes mellitus (IDDM) – has a childhood onset. In this type, the insulin secreting B-cells of the islets of Langerhans are incapable of producing insulin as a consequence of auto immune attack of these cells resulting in their destruction (Pazdro and Burgess, 2010; Zhang and Eisenbarth, 2011). The autoimmune destruction of Islets in diabetics was linked to the presence of Islet cell-specific antibodies, excessive interleukin production by monokines; a deranged T-cell mediated immunoregulatory system and activation of antigens targeting the Islets cells (Ozougwu *et al.*, 2013). Therefore, daily doses of insulin are given to these patients to maintain euglycemia and thus preventing life threatening complications (Padgett *et al.*, 2013). Type 1 diabetes accounts for 5-10% of all diabetic cases globally. Type 1 DM can also exist idiopathically with no known cause in the absence of autoimmune destruction of the B-cells (MacLarty *et al.*, 1990).

2.2.2 Type 2 DM

Type 2 diabetes mellitus (T2 DM) is also known as noninsulin dependent diabetes mellitus (NIDDM) – the β-cells of the pancreas produce detectable amounts of insulin. However, the problem arises in the recognition of the insulin by its receptors in the target organs (insulin resistance) resulting in disturbed insulin intracellular signalling cascades (Pan *et al.*, 2010; Amod *et al.*, 2012). Insulin resistance (IR) has been described as the failure of insulin to execute normal biological signals as expected from its blood concentration, and as a consequence diminishes the rate of normal fuel metabolism (Kaku, 2012). IR is believed to be the first pathological defect noted in T2 DM followed by beta cell dysfunction as a

complication that arises due to compensatory response mechanisms from the pancreas (Kahn, 2003; Kaku, 2012).

Type 2 DM is the most common form accounting for 90-95% of all cases globally and it usually develops later in life during adulthood (Eze *et al.*, 2012). It is marked by 4 principal metabolic features which include; central obesity (obesity intensifies the progression of insulin resistance), impaired insulin action, insulin secretory dysfunction and increased endogenous glucose output (Weyer *et al.*, 1999; Scheen, 2003). The causes of this type of diabetes are multifactorial and revolve around genetic alterations and environmental factors (Tuoilehto *et al.*, 2001; Ozougwu *et al.*, 2013). Experts have reported a higher concordance of type 2 DM in monozygotic twins and a 2.4 fold risk for an individual from a family with a history of DM with the highest risk noted in first degree relatives of diabetic patients (Stumvoll *et al.*, 2005; Leahy, 2005). A strong emphasis on genetic factors being the chief cause of DM has been linked to the highest incidence of diabetes in the Pima Indians, the Finnish and the Arab ethnic groups in comparison to the Caucasians (Ozougwu *et al.*, 2013). Associations of environmental and lifestyle factors have also been implicated in the pathogenesis of diabetes. Dietary constituents, smoking, alcohol intake, advanced age, consumption of certain corticosteroid drugs, growth hormones and lack of exercise are detrimental effects that affect insulin sensitivity hence negatively impacting glucose homeostasis (Kaku, 2010; Pazdro and Burgess, 2010).

2.2.3   Other aetiological classification of DM

- Gestational diabetes mellitus
- Genetic defects of β cell function
- Genetic defects in insulin action e.g. Lipoatropic diabetes
- Diseases of the exocrine pancreas e.g. Pancreatitis
- Endocrinopathies
- Drug and chemical induced diabetes
- Infection induced diabetes e.g. CMV (Diabetes Care, 2011).

2.2.4   Diagnosis Criteria

In 1999, the world health organisation (WHO) reached an accord in the diagnosis criteria for DM. Inclusion of impaired fasting glucose and impaired glucose tolerance was recognised because several reports indicated that 7%-30% of people with impaired glucose homeostasis later developed diabetes within a year (Stumvoll *et al.*, 2005; van Dieren *et al.*, 2010). Impaired fasting glucose (IFG) and the impaired glucose tolerance (IGT) stages are

intermediate stages between normoglycemia and the diabetic stages (Table 2.1). These intermediate stages are both referred to as pre-diabetic stages. Epidemiological studies have also indicated that though IGF and IFG are risk factors for the development of diabetes mellitus, not all patients diagnosed with pre-diabetes would eventually be diabetic. Development of diabetes from the pre-diabetic stages was associated with other risk factors such as obesity, hypertension and dyslipidemia whereas proper lifestyle interventions such as increased physical activity and healthy dietary practices have been shown to interrupt the development of diabetes (American Diabetes Association, 2010). Recommended lifestyle changes in pre-diabetics are said to reduce the risk of diabetes emergence by 28-58%, therefore critical changes are important (Nolan *et al.*, 2011).

Table 2.1: Diagnosis criteria

Stage	Fasting Blood Glucose (FBG) (mmol/l)	Postpradrial Glucose in plasma (mmol/l)
Diabetes mellitus	FBG ≥ 7.0	2hr OGTT value ≥ 11.1
IGT	FBG < 7.0	2hr OGTT ≥ 7.8 < 11.1
IFG	FBG ≥ 6.1 ≤ 7.0	2hr OGTT < 7.8

Abbreviations: IGT- Impaired glucose tolerance; IFG- Impaired fasting glucose (Diabetes Care, 2011)
Fasting glucose indicates to absence of caloric intake in preceding 8hrs, OGTT- oral glucose tolerance test where patients ingest 75g of anhydrous glucose dissolved in water.

2.2.4.1 Signs and symptoms of DM

- Polyuria - is a term used to describe the excessive urine flow rate that is caused by glucose osmotic diuresis.
- Polyphagia - refers to the uncontrolled hunger feeling in diabetics as a result of the inadequate glucose that is needed in energy (ATP) production.
- Polydipsia – describes the continuous thirst feeling. Glucosuria has been linked to increased polyuria in an attempt to reset the osmolality caused by increased glucose, vasopressin (diuretic hormone) levels are in turn increased in diabetics, it acts by preventing the reabsorption of water back in circulation, as a consequence more water is excreted, decreasing the circulatory volume. In response to excess fluid loss (polyuria), diabetics have increased thirst (Bankir *et al.*, 2001).
- Numbness and dizziness
- Retarded wound healing
- Blurred vision
- Frequent infections

- Weight loss (Riaz, 2009; Unwin *et al.*, 2011).

2.3 DM and its epidemiology

The prevalence of DM has increased world wide and 25% of the world population is believed to be suffering from DM (Oyagbemi *et al.*, 2014). DM has become a major public health burden that is reported to become the 7th leading cause of mortality by the year 2030 (Arise *et al.*, 2014). It has also been predicted that 552 million people will be diabetics by 2030 (Whiting *et al.*, 2011). Currently, it is estimated that 382 million people are diabetics (Sen *et al.*, 2015). The rate of the incidence of DM is so alarming that reports project that 1 in 10 non-diabetic adults will become a diabetic patient by the year 2030 (Arise *et al.*, 2014). In 2004, studies done in middle income countries reported that DM was responsible for an estimated 0.5 million deaths, which is approximately 2.1% of the total deaths that occurred whereas in high income countries 0.2 million deaths were due to DM, accounting for 2.8% of the total deaths, reflecting the great burden of DM in developing countries (International Diabetes Federation, 2011).

In the past decades, the prevalence of type 2 DM was reported to be generally low in Africa and deaths caused by DM were insignificant, mainly because of the consumption of natural unprocessed plant foods. However, due to changes in the dietary trends and lifestyle, increasing figures of mortality in African societies due to DM are becoming noticeable (Udenta *et al.*, 2014). A 2.5 fold increase in DM prevalence is expected in the developing countries by the years 2020-2025 (Amine *et al.*, 2003). In Sub Saharan countries, 10.2 million people are reported to have type 2 DM. The figures are expected to reach 18.7 million by 2025 (Abo *et al.*, 2008; Levitt, 2008). Nigeria tops the list of 5 countries greatly affected by DM in the Sub-Saharan region with 3.9 million diabetic people, followed by South Africa with 2.6 million, Ethopia with 1.8 million, Tanzania and Congo with 1.7 million and 1.6 million diabetics respectively (International Diabetes Federation, 2014). In addition, global epidemiological studies reported a greater prevalence of diabetes mellitus in women than in males substantially attributed to physical inactivity and obesity in females (van Dieren *et al.*, 2010).

2.3.1 Epidemiology of Type 1 DM

Type 1 DM has the highest prevalence and incidence in European populations, with an overall rate of increase of 3-4% (Nerup and Pociot, 2001; Gale, 2002). Non-European populations around the world have relatively low incidence of type 1 DM (Whiting *et al.*, 2011). The highest incidence has been noted in the Scandinavian countries and in north-

west Europe, with Finland, Sweden and Norway in the top 3 of the countries significantly affected (Green and Pattersson, 2001). Type1 DM has a childhood onset and studies have reported that 25 juveniles (< 15 years old) in a 100 000 population in Nordic countries have type 1 DM. According to the Swedish Childhood Diabetes studies, a large number of individuals of European origin have a form of beta cell autoimmunity that is mainly propelled by environmental factors and genetic factors to a lesser extent (Berhan *et al.*, 2011).

2.3.2 The economic and Health and Wellness burden of DM

DM is a challenging health problem that has raised a lot of concern globally. In high income countries, it is presumed to be the 4th or 5th leading cause of mortality (Whiting *et al.*, 2011). Current studies highlight the rising prevalence in poorer low-income countries and the burden will continue to be severe if not urgently addressed (Shaw *et al.*, 2010; Whiting *et al.*, 2011). As a result of increased disability due to DM complications, a country's economy is significantly and negatively affected. Affected patients to some extent will not be able to render maximum services to their respective organisations leading to diminished production output. For instance, in the USA, diabetic patients are 15 times more prone to lower limb amputation than non-diabetics (Amos *et al.*, 1997). Diabetic retinopathy is one of the leading causes of blindness between 20-74 years age groups thus inflicting a burden on global expenditure (Nolan *et al.*, 2011). Statistical reports in 2007 revealed the global cost of diabetes to be approximately US$ 174 billion and for every $5, $1 is spend on a diabetes patient (Freeman, 2010). In Sub Saharan Africa, $ 67, 3 billion is spent annually and the USA spends approximately 50% of the total annual global expenditure. Lowest expenditure figures are noted in the least developing countries where the treatment and management of DM is crippled (Oyagbemi *et al.*, 2014).

2.4 Oxidative Stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radicals that are produced during normal aerobic respiration in the mitochondria (Ray *et al.*, 2012). Free radicals are described as reactive molecular species that have unpaired electrons in their outer atomic orbitals. ROS is a broad term used to categorise molecules derived from incomplete oxygen reduction reactions whilst RNS comprises of products from nitrogen oxidation reactions (Wagener and Dekker, 2009). Examples of ROS include superoxide ion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the hydroxyl radical ($HO^{\cdot}$) whereas nitric oxide (NO), nitroxyl (NO^{-}), S-nitrosothiol (RSNO) peroxtnitrite ($OONO^-$) are types of RNS (Araki and Nishikawa, 2010; Pitocco *et al.*, 2010). Oxygen is the principal

substrate utilised during cellular respiration. Not all of the oxygen inhaled is harnessed in the production of ATP; rather, about 5% of the inhaled oxygen reacts with different molecules resulting in the formation of these ROS/RNS (Berk, 2007). When produced during normal homeostasis, these free radicals participate in cellular signalling, cell growth, apoptosis, defence against pathogens and in regulatory pathways (Mittler, 2002; Wagener and Dekker, 2009).

If an imbalance arises between the production of ROS/RNS and the ability of antioxidants in biological systems to degrade or to remove these free radicals, where the imbalance favours free radical production, oxidative stress will subsequently result as illustrated in Figure 2.2 (Araki and Nishikawa, 2010). Oxidative stress is noted in various pathological states of different disorders displayed by the overwhelmed production of free radicals and depleted production of antioxidants (Sies, 1996; Niedowicz and Dalake, 2005). In an event of overt oxidative stress, cellular components like proteins, lipids and DNA are irreversibly modified, ultimately resulting in pathogenesis of various pathologies (Pitocco *et al.*, 2010).
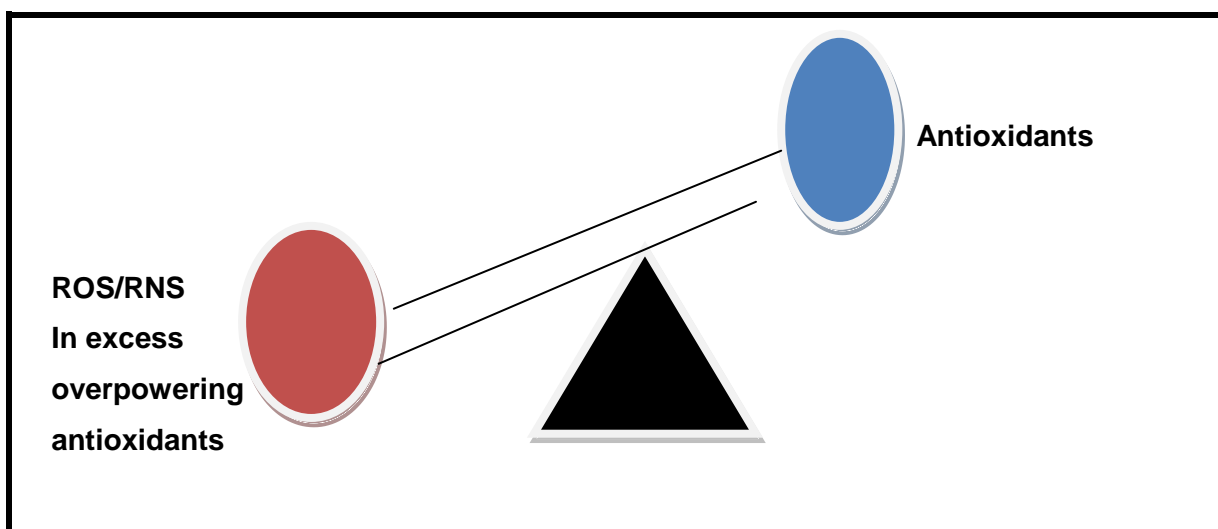
Figure 2.2: **Schematic diagram of the relationship between ROS/RNS and antioxidants in oxidative stress**

2.4.1 Implication of oxidative stress in the progression of diabetic complications

The chronic sustained hyperglycemic condition/ state in diabetes has been attributed as the major factor that triggers the development of both acute and long term changes in the

cellular metabolism of different molecules (Giacco and Brownlee, 2010; Pan *et al.*, 2010). Altered metabolism of macromolecules ultimately triggers excessive formation of free radicals via different pathways as a result speeding up the development of diabetic complications (Wagener and Dekker, 2009). ROS are incriminated in the disruption of glucose transport systems and in the defective receptor - ligand sensitivity between insulin and its receptor, giving rise to insulin resistance (Radoi *et al.*, 2010). It has been strongly suggested that oxidative stress plays a key role in the development of diabetes vascular complications. Malignant transformations of tissues as a consequence of OS are mostly seen in the eyes, nervous system and the kidneys (Pazdro and Burgess, 2010). Several pathways have been established that link the role of oxidative stress in promoting the pathogenesis of diabetic complications. The growing body of evidence has reported that hyperglycemia causes tissue injury via 4 major mechanisms: (1) increased glucose flux through the polyol pathway; (2) increased intracellular formation of AGEs (advanced glycation end products); (3) activation of the protein kinase (PK) C pathway and (4) increased activity of the hexosamine pathway. Scientific evidence has shown that all four mechanisms are triggered by a particular upstream event that points to the overproduction of reactive oxygen species by the mitochondria (Rolo and Palmeira, 2006; Giacco and Brownlee, 2010).

2.4.2   Advanced glycation endproducts formation (AGE) s

Progression of oxidative tissue damage occurs at a faster rate in patients with poor metabolic control of hyperglycemia, hyperglycemia impels the formation of AGEs and the presence of AGEs has been associated with the severity of diabetic complications (Musabayane, 2012). Advanced glycation endproducts; are heterogeneous group of molecules formed in the absence of enzymatic catalysis in a reaction termed the Maillard reaction (Radoi *et al.*, 2010). The first step in the glycation pathway is the slow reversible nucleophilic reaction between the carbonyl groups (aldehydes/ ketones) of reducing sugars or monosaccharides with the amine groups of proteins, nucleic acid or lipids (Lehmann and Scheleicher, 2000). The outcome of the reaction is the formation of highly reversible products known as the Schiff bases (Soldatos and Cooper, 2008; Rains and Jain, 2011). Spontaneous rearrangement reactions of the Schiff bases follows over a period of days to weeks forming more stable compounds termed Amadori products. Further rearrangement and oxidation of the latter compound takes place resulting in AGEs (Lehmann and Schleicher, 2000; Singh *et al.*, 2001; Ahmed, 2004; Peppa *et al.*, 2003; Rains and Jain, 2011; Yamagishi, 2012; Arsov *et al.*, 2014). The series of reactions that take place in the formation of AGEs are demonstrated in Figure 2.3.

C=O + H₂N — PROTEIN   H - C = N— PROTEIN   H₂ - C— NH — PROTEIN

Represented as chemical structures:

$C=O \quad + \quad H_2N - \text{PROTEIN} \qquad H-C=N- \text{PROTEIN} \qquad H_2-C-NH - \text{PROTEIN}$

| Glucose | Protein | Schiff base | Amadori product |

$$H_2-C-NH - \text{PROTEIN}$$
Dicarbonyl intermediate such as protein-bound 1,4 deoxyglucosone

AGE — PROTEIN
and / or
PROTEIN — AGE — PROTEIN

Advanced Glycation Endproducts (AGE)
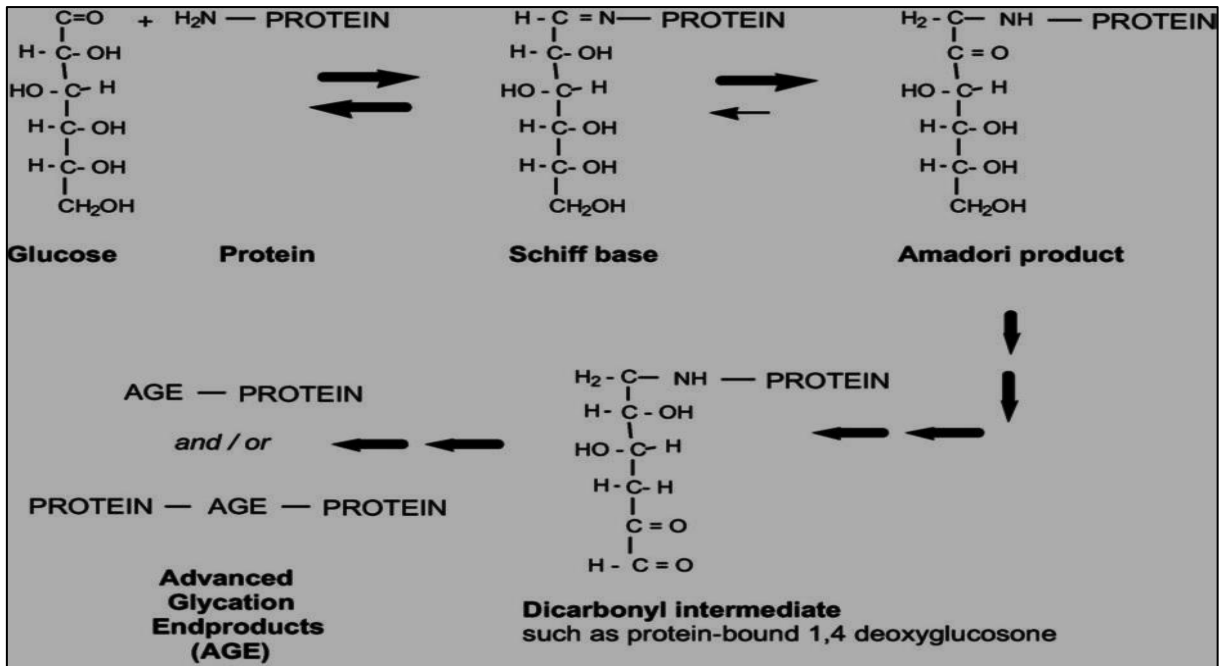
Figure 2.3: The classical pathway of protein glycation by glucose leading to AGEs formation (Peyroux and Sternberg, 2006)

AGEs principally target long lived connective tissue proteins like type IV collagen, tubulin, plasminogen activator 1 and fibrinogen forming irreversible cross linkages with them (Singh *et al.*, 2001). The formed cross-links later develop into tough fibres which stiffen the blood vessels, diminishing arterial and myocardial compliance as well as increasing diastolic dysfunction and systolic hypertension (Goh and Cooper, 2011; Arsov, 2014). Modification of the cellular matrix of the basement membranes by AGEs was also implicated in the severity of tissue damage in diabetic nephropathy (Singh *et al.*, 2001b; Peppa *et al.*, 2003).

Signalling of AGEs involves their interaction with their cell surface receptors called the receptor of advanced glycation end products (RAGE). Binding of AGEs to RAGE triggers the activation of NADPH oxidase system, cytokine release and free radical activity further enhance tissue damage (Tiganis, 2011). The production and translocation of NF-ĸβ via the Ras-MAPK pathway has been extensively studied as the product of the association of AGEs and RAGE. NF-ĸβ is a transcriptional factor that modulates the transcription of vascular endothelial growth factor (VEGF) which is a mitogen that stimulates the mitosis of endothelial cells therefore increasing their permeability and the progression of vascular diabetic complications (Radoi *et al.*, 2010). Experimental evidence reported a direct proportional relationship between the levels of AGEs and the severity of diabetic complications. Furthermore, it has been indicated that AGEs alter enzymatic activities, cause protein fragmentation, decrease ligand binding capacity and immunogenicity alterations (Radoi *et al.*,

2010). Pentosidine, carboxymethyl-lysine (CML, a product of glycoxidation), imadazolones and pyrraline are predominant types of endogenous AGEs that have been studied extensively (Ahmed, 2004).

2.4.3 The increased polyol pathway flux

The polyol pathway, also known as the aldose reductase (AR) pathway, is activated when there is an upsurge in the concentration of glucose in cells (Lorenzi, 2007). The pathway utilises keto-reductase enzymes to catalyse the reduction of carbonyl compounds of sugars into their respective polyols (Giacco and Brownlee, 2010). In the course of normal metabolism, AR facilitates the reduction of reducing sugars into inactive alcohols, however if cells encounter excess levels of glucose, AR as the rate limiting enzyme converts glucose into sorbitol. The conversion depends on NADPH as the cofactor (Brownlee, 2005). Oxidation of sorbitol into fructose by sorbitol dehydrogenase (SDH) follows with NAD^+ as the cofactor (Giacco and Brownlee, 2010). The most feasible explanation on how an increased polyol flux causes detrimental tissue damage is the depletion of NADPH in the aldo-keto reaction. NADPH is an important cofactor of glutathione reductase in reactions that trigger the generation of glutathione (GSH) from its oxidised form GSSH. Over consumption of NADPH in the aldo- keto reaction deprives glutathione reductase (GR) of its cofactor resulting in attenuated GSH production, a critical antioxidant that scavenges free radicals and the ROS. Deficiency in GSH further depletes antioxidant defence systems leading to oxidative stress (Chung *et al.*, 2003; Brownlee, 2005; Lorenzi, 2007; Giacco and Brownlee, 2010). In addition, phosphorylation of fructose to fructose-3-phosphate takes place, the phosphorylated fructose molecules undergo degradation resulting in the formation of 3-deoxyglucosone. These two products of fructose phosphorylation are strong glycosylating compounds that glycosylate molecules giving rise to AGEs precursors (Chung *et al.*, 2003). The oxidation reaction of sorbitol to fructose by (sorbitol dehydrogenase) SDH has been documented as the channel that favours ROS formation. This is because in the reaction, NAD^+ is reduced to NADH. NADH is a substrate of NADH oxidase in one of the pathways that forms ROS (Chung *et al.*, 2003). The increased polyol flux oxidative damage was greatly indicated in the retina of the diabetic mice that overexpressed AR. Over expression of AR and sorbitol in diabetic mice also increased atherosclerosis and diabetic lesion respectively (Giacco and Brownle, 2010).

2.4.4 Mitochondrial production of superoxide anion

The mitochondria are cellular organelles housed in the cytoplasmic area that are important in the survival and normal functioning of cells (Brand *et al.*, 2004). The mitochondria are made up of two distinct areas that are surrounded by inner and outer membranes. These membranes are important in the maintenance of normal electrochemical gradients (Echtay, 2007). During normal respiration in the electro transport chain (ETC), electrons from reduced substrates pass through different redox centres grouped into four complexes; complex I (NADH dehydrogenase), complex II, complex III (ubiquinone cytochrome C oxidoreductase) and complex IV (cytochrome oxidase). The overall effect of the passage of electrons is the pumping of protons (H^+) into the inner membrane space. This movement of H^+ generates a proton motive force across the inner membrane that translocates the protons back into the matrix. As protons move back into the matrix, complex V also termed ATP synthase is driven leading to the generation of ATP. Oxygen molecules are the final acceptors of these electrons resulting in the formation of H_2O and the superoxide anions (0, 15% of oxygen is converted to superoxide ions), very reactive precursors of ROS (Echtay, 2007, Pazdro and Burgess, 2010). Although the superoxide anion is produced during normal respiration, it has been identified that evolution has mechanisms to counteract development of OS because of the availability of endogenous antioxidants such as Manganese superoxide dismutase (MnSOD). MnSOD is a mitochondrial-derived enzyme that dismutates superoxide anions into hydrogen peroxide (H_2O_2). Hydrogen peroxide is in turn converted to water and oxygen by the enzyme glutathione peroxidase (GPx) (Jezek and Hlavata, 2005; Grivennikova and Vinogradov, 2006; Echtay, 2007; Buettner, 2011).

Prolonged exposure of cells to high glucose concentrations favours the oxidation of glucose in the form of pyruvate. Oxidation of pyruvate increases the influx of electron donors NADH and FADH2 into the ETC increasing the voltage gradient across the mitochondrial membranes. The electrons being transferred bypass complex III to coenzyme Q which will then donate them to oxygen molecules generating excessive amounts of superoxide anions (Giacco and Brownlee, 2010; Kashihara *et al.*, 2010). Production of superoxide anions in this side reaction overpowers the ability of SOD to degrade thus empowering oxidising ability of superoxide anions on proteins, DNA and lipids inflicting permanent oxidative changes. Moreover, hydrogen peroxide generated in the reaction has been associated with the production of hydroxyl radicals in the presence of ferrous ions. Hydroxyl radicals are powerful free radicals that potentially cause tissue damage (Genova *et al.*, 2001; Brand *et al.*, 2004). Despite the implication of mitochondrial superoxide production in diabetes, it has been reported that mitochondria are the main source of ROS and involved in the aetiology of diseases such as cancer, aging, ischemia, obesity, Alzheimer's and Parkinson's diseases (Grivennikova and Vinogradov, 2006; Schwarzlander, 2012).

2.4.5 Increased protein kinase C activation

Protein kinase C is a group of enzymes made up of 11 isoforms that belong to serine / threonine related protein kinases family (Pinton, 2013). The enzymes take part in the phosphorylation of the target proteins. Activation of PKCs isoforms requires the presence of calcium ions, phosphatidylserine (PS) and diacylglycerol (DAG) (Giacco and Brownlee, 2010). Over activity of the PKC pathway acts as an alternative route for the formation of hyperglycemia-induced ROS. Enhanced levels of glucose speeds up the synthesis of DAG via the triose phosphate in which the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited by ROS. Inhibition of the GAPDH favours the formation of a DAG precursor (Avignon and Sultan, 2006; Giacco and Brownlee, 2010). Hyperglycemia also increases the levels of dihydroxyacetone phosphate which is reduced to glycerol-3-phosphate. Glycerol-3-phosphate conjugates with fatty acids thus increasing the de novo synthesis of DAG which further activates the PKCs again (Schimitz and Biden, 2008). PKC isoforms control several cellular signals like the NADPH oxidase and NF- κβ, the two have major roles in the events that trigger ROS production. Activation of PKCs by glucose further contributes to the abnormal increase in vascular permeability as a consequence of vascular endothelial growth factor (VEGF) overexpression, thickening of the basement membrane, apoptosis and expansion of extracellular matrix (Evcimen and King, 2007; Fernández-Mejía and Lazo-de-la-Vega- Monroy, 2013).

2.5 Antioxidants: Classification and their roles in combating oxidative stress

An antioxidant is defined as, "any substance that when present in low concentration compared to that of the oxidisable substrate significantly delays or inhibits the oxidation of that substrate and the final outcome is its oxidation" (Young and Woodside, 2001).

Antioxidant is a broad term also used to characterise;
- Enzymes that degrade free radicals (catalase, SOD and glutathione peroxidase).
- Proteins that have the ability to bind to metals that initiate free radical generation for instance transferrin.
- Exogenous antioxidants (vitamin C, vitamin E, flavonoids, carotenoids, alpha tocopherol and non-enzymatic GSH (Rahimi *et al*, 2005; Valko *et al*, 2007).

The antioxidant properties of the above mentioned compounds are derived from their ability to:
- Break the reaction chains of free radicals e.g. during lipid peroxidation

- Sequestrate transition metal ions
- Repair damaged molecules
- Scavenge radicals (uric acid, ascorbic acid and albumin) (Valko *et al.,* 2007; Noori, 2012; Gupta *et al.*, 2014).

Antioxidants can be further grouped into endogenous sources (SOD, catalase, GPx) and exogenous sources (derived from dietary sources) (Noori, 2012). Constant replenishing of the endogenous antioxidant pool is essential because they are inevitably consumed during free radical attack. The shortage is counterbalanced by antioxidant supplementation from plant materials. A growing body of evidence has shown that plants possess phytochemicals that exhibit strong antioxidant activities (Echty, 2007; Pham-Huy *et al.*, 2008). The antioxidant compounds extracted from plants that have been associated with high antioxidant powers include flavanoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, alkaloids, terpernoids, polyphenols and isocatechins (Birden, 2012; Noori, 2012). For this reason adequate intake of exogenous antioxidants play a fundamental role in the prevention and treatment of various pathologies which include diabetes, neurodegenerative diseases, pulmonary disorders, aging related diseases and cancer (Sen *et al.*, 2010; Veskoukis *et al.,* 2012). Studies done to compare the antioxidant capacity of non-diabetics and type 1 diabetic subjects reported a 16% lower capacity in type 1 patients (Rohimi *et al.*, 2005).

Accumulation of reactive oxygen species (ROS) and their ability to oxidise biological molecules is known to contribute to an increased rate of cell shrinkage, chromatin condensation, DNA fragmentation as well as compromised signalling mechanisms (Habib, 2013). Also, lack of appropriate defence strategies to revert the uncontrolled generation of reactive oxygen species leads to the development of adverse diabetic complications. The availability of adequate supply of antioxidants greatly intercepts the oxidising effects of free radicals.

Table 2.2: Exogenous antioxidants identified in dietary sources

Antioxidant	Sources	References
Ascorbic acid/ Vitamin C	Citrus fruits, vegetables, green tea	Romero *et al*, 2013
Vitamin E/ tocopherol	Wheat, nuts and vegetables	Pham-Huy *et a*l, 2008
Vitamin A/ carotenoids	Carrots, leafy vegetables	
Flavanoids	Grapes, apples, green tea, cabbage and beans	Bouayed and Bohn, 2012 Pham-Huy *et al.*, 2008
Polyphenols	Apples, onions, whole grain products, potatoes	Bouayed and Bohn, 2012

2.6 Diabetic Kidney Disease (DKD)

Diabetic kidney disease (DKD) which is a term used interchangeably as diabetic nephropathy is a major complication of diabetes that affects approximately 20-40% of diabetic patients globally (Dronovalli *et al.*, 2008). DKD is categorised as a microvascular complication that develops into end stage renal disease (ESRD) and cardiovascular disease as a result, increasing the mortality rate of diabetes (Gross *et al.*, 2005; Johansen *et al.*, 2005; Dang *et al.*, 2010). Diabetic kidney disease exists in several phases of development that are initiated by different mechanisms. The initial marker of diabetic kidney disease in the absence of other renal diseases, is the leakage of albumin to the urine also referred to as urine albumin excretion (UAE). Two distinct levels of albuminuria are used to categorise the stages of DKD progression: microalbuminuria (> 20 µg/min and < 199 µg/min) and macroalbuminuria (≥ 200µg/min) (Gross *et al.*, 2005; Elmarakby and Sullivan, 2012). Recently, the need of including routine glomerular filtration rate (GFR) screening in the diagnosis of DKD has been recognised because it was reported that some patients present with normal albumin levels in the urine but with decreased GFR. Therefore, the two provide a realistic picture of the progression of diabetic nephropathy (Zelmanovitz *et al.*, 2009).

2.6.1   Functional and structural characteristics that mark renal disease in diabetic patients

2.6.1.1 Pathophysiology

The hyperglycaemic state in diabetes causes structural changes in the kidney morphology (Dronovalli *et al.*, 2008). The first structural changes observed is the thickening of the glomerular and tubular basal membranes' width (glomerular hypertrophy) (Gross *et al.*, 2005). GFR decreases in response to the expansion of mesangial matrix. Expansion of the mesangial matrix manifests as diffuse which is known as diabetic glomerulosclerosis or in particular areas referred to nodular glomerulosclerosis. The kidney will finally lose its structural and functional integrity due to the formation of nodules and lesions. Hyalination of glomerular arterioles occur leading to arteriosclerosis and microaneurysm. Glomerulosclerosis furthermore involves disruption of podocyte architecture causing a decrease in GFR (Gross *et al.*, 2005; Zelmanovitz *et al.*, 2009). The functional changes in DKD that accompany structural changes in the kidney include; increased tubule-glomerular feedback, increase in systemic blood pressure, decreased creatinine clearance, proteinuria and glomerular hyperperfusion (Arora and Singh, 2013).

2.6.2   Pathways implicated in the development of DKD.

2.6.2.1 Haemodynamic factors

Poor glycemic control together with arterial hypertension, are significant risk factors that lead to the onset as well as progression of DKD (Ha and Kim, 1999). Early stages of DKD are characterised by increased renal perfusion and glomerular hyper filtration as a consequence of the reduction in the resistance of both the afferent and efferent glomerular arterioles (Zelmanovitz *et al.*, 2009). Increase in renal perfusion is linked to high levels of endothelin, which is a vasoconstrictor; its enhanced levels are directly proportional to the extent of UAE. Disturbances in the autoregulatory mechanisms of renal perfusion impairs the normal movement of albumin from capillaries to the glomerulus resulting in the expansion of the mesangial membrane, thickening of the basement membrane as well as podocyte damage (Dronovalli *et al.*, 2008).

Many factors have been implicated in the defective autoregulatory system of renal perfusion (Zelmanovitz *et al.*, 2009). These factors include nitric oxide, prostanoids, the vascular endothelial growth factor (VEGF) and the angiotensin system expedite albumin leakage from the glomerular capillaries into the urine (Dronovalli *et al.*, 2008; Arora and Singh, 2013). The

accumulation of albumin in the urine systematically activates various inflammatory pathways through tubular cells. Renal hyperperfusion exerts mechanical stress on the matrix triggering the release of cytokines (interleukin 1, 6 and 18 and tumor necrosis factor) that causes protein accumulation from the extracellular matrix leading to further mesangial expansion (Zelmanovitz *et al.*, 2009).

2.6.2.2 The renin angiotensin system

Angiotensin II is a vasoactive hormone that causes glomerular hypertension by stimulating the transforming growth factor (TGF-β1). TGF-β1 is profibrotic cytokine that increases systemic and intraglomerular pressure that will eventually cause mesangial expansion by triggering the production of collagens, laminin and fibronectin, UAE and stimulation of VEGF expression (Zelmanovitz et al., 2009). VEGF is a vasodilator that not only increases the permeability of the glomerular capillaries to proteins but also leads to the production of NO (Arora and Singh, 2013). It has been reported that VEGF triggers the production of α 3 chain of collagen IV. Increased levels of α 3 chain further promote the thickening of the GBM (Dronovalli *et al.*, 2008).

2.6.3   Oxidative stress as a major factor in the development of diabetic nephropathy

It has been clearly demonstrated that oxidative stress plays a crucial role in impairing normal kidney function in diabetes (Tavafi, 2013). Hyperglycemia disturbs normal cellular processes through induction of oxidative stress and inflammation. Generation of ROS in the renal tissue has been shown to be related to the modification of the extracellular matrix, vasoconstriction, overgrowth of the vascular smooth muscles, endothelial dysfunction and sodium imbalance (Elmarakby and Sullivan, 2012). The relationship between oxidative stress and cytokine production has been established as ROS act as second messengers that activate various transcription factors that encode for cytokine genes. For example NF-κβ is a transcription factor produced via the Ras-MAPK pathway as a result of the association of AGEs and RAGE (Figure 2.4). NF-κβ plays a key role in the events that lead mesangial cell activation (Radoi *et al.*, 2011; Elmarakby and Sullivan, 2012). The overproduction of the AGEs in renal tissues has been reported as the chief pathway that exerts tissue damage as a result of the formation of crosslinks within the renal matrix. The extensive damage was ascribed to the role the renal system plays in the final clearance of these AGEs. Other studies reported that long-lived AGEs in renal tissue cripple the antioxidant defence functions of CuSOD and MnSOD thus empowering ROS damage (Forbes *et al.*, 2008). In addition, the effects of abnormal mitochondrial electron transport chain, auto oxidation of glucose, activation of the

polyol, aldo-keto reductase and PKC pathways have also been strongly emphasised in the pathogenesis of diabetic nephropathy as shown in Fig 2.4. Induction of apoptosis in renal tissue as consequence ROS production has been shown by different studies (Kashihara *et al.*, 2010; Forbes and Cooper, 2012). Other studies done in STZ induced animal subjects indicated that the inhibition of OS was successful in attempts done to reverse manifestations of diabetic nephropathy (Elmarakby and Sullivan, 2012).
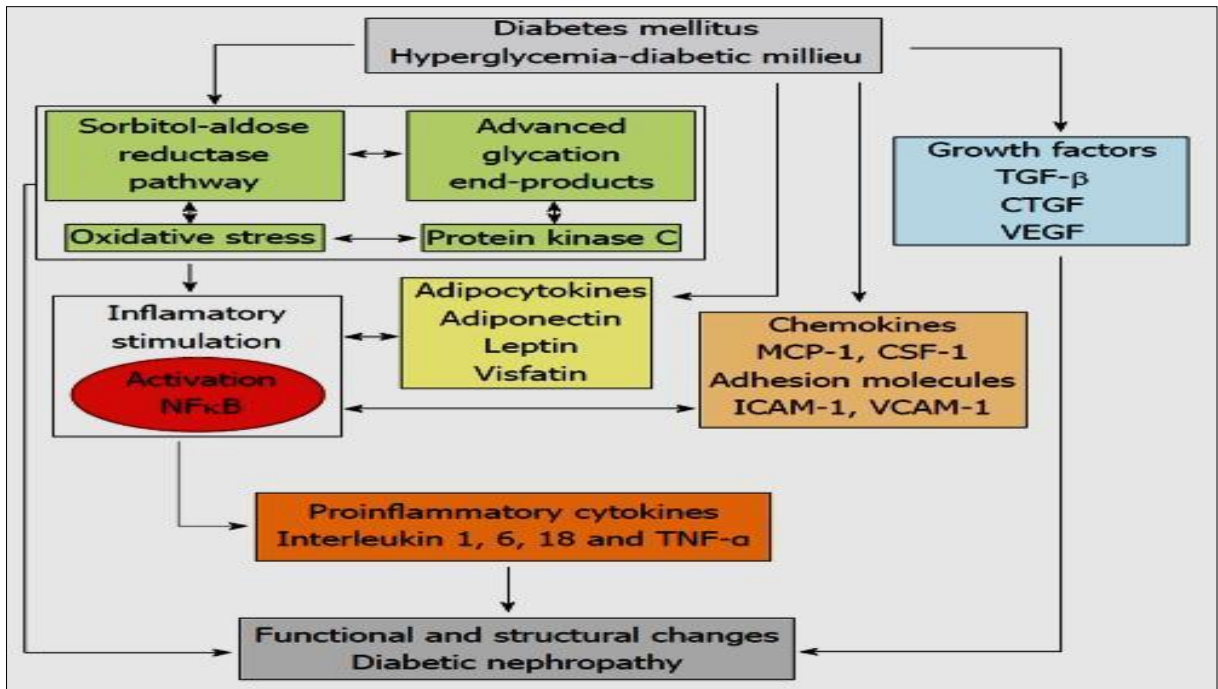
Figure 2.4: Schematic drawing of hemodynamic and metabolic factors that trigger DKD (Luis-Rodriguez *et al.,* 2012)

2.7 Diabetic liver disease

The prevalence of diabetic hepatic disease in diabetic patients has been estimated to lie between 17 and 100 % and association of the disease has been reported in type 2 obese subjects (Al-Hussaini, 2012). Hepatic insulin resistance is the major metabolic factor reported in diabetics that leads to the development of non alcoholic fatty liver disease (NAFLD). NAFLD is used to describe different liver pathologies which include benign conditions, hepatic glycogenosis and non-alcoholic steatohepatitis. Diabetic hepatologies are frequently marked by the following clinical and metabolic features: abnormal liver function tests, abdominal discomfort, nausea, vomiting, hepatomegaly and increased glycogen storage in hepatocytes (Fridell *et al.*, 2007; Saadi, 2012). In type 1 diabetes, insulin deficiency negatively influences glucokinase levels and hence lowering the glucose uptake in hepatic

cells. In response to the disturbed glucose metabolism, hepatic glucagon levels increases thus initiating glycogenolysis and gluconeogenesis (Al-Hussaini, 2012). It has been shown that decreased levels of insulin will cause glucose output by the liver to increase. Over insulization (as a result of insulin therapy) in type 1-diabetes in turn causes hepatic glycogenosis where there is a marked increase of glycogen molecules in hepatocytes. Hepatic glycogenosis is a reversible condition that is treated by glycemic control (Messeri *et al.*, 2012; Jardim *et al.*, 2013). ROS have been implicated in the emergence of diabetic hepatopathies to a lesser extent. The involvement of ROS has been linked to lipid peroxidation of hepatocytes' membranes and apoptosis of hepatocytes (Frances *et al.*, 2010).

2.8 . Treatment and management of DM
2.8.1   Diet and lifestyle modification

An early diagnosis of a hyperglycaemic state and a comprehensive approach in the maintenance of normoglycemia is linked to favourable long term glycemic control. Patient education and life style modifications greatly improve the prognosis of diabetic complications, for example cardio-vascular diseases (CVD) complications can be reduced by 70-80% (Nathan *et al.*, 2009; Nathan and Edic, 2014). Diabetic patients and their families are the best people to provide supportive care and management of DM as it is affected mainly by daily fluctuations in environmental stress, diet and infections (Imam, 2012).

Researchers have reported that oral anti-diabetic drugs (OADs) together with lifestyle changes can provide both short and long term beneficial effects in diabetic patients, hence the need to educate pre-diabetics and diabetic patients on the importance of proper care and management of DM (Nathan *et al.*, 2009). Emphasis should be put on the appropriate dietary and lifestyle changes (Puder and Keller, 1989). Various clinical trials proved that type 2 DM can be prevented by modifying dietary and lifestyle practices (Hall *et al.*, 2011; Hu, 2011). Diet and exercise are the primary prevention strategies of the pathogenesis of DM as well as other chronic diseases (Joint WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases, 2002). A high energy expenditure achieved through physical activity in diabetic patients relatively decreases insulin resistance while increasing glucose tolerance at the same time. Physical activity has a great impact on the amount of fat, muscle and bone tissue therefore it determines the extent of one being overweight and obese. A weight loss of ≥ 4 kilograms in diabetics ameliorates hypergylcemic complications (Marliss and Vranic, 2002; Nathan *et al.*, 2009). A major obstacle detected in the role of physical

activity interventions is the emergence of musculoskeletal injuries and foot ulcers experienced by diabetic patients.

Unhealthy dietary habits that emerge due to rapid globalisation are the chief causes of non-communicable diseases aetiology (Steyn and Labadarios, 2008). In diabetics, a high trans-fat and carbohydrate diet are major factors that exacerbate development of hyperglycemia. It is thus important to enlighten diabetic patients on the quantity and quality of carbohydrates to be consumed (Caloric count) so that they can be aware of the exact amount of insulin to administer (Imam, 2012). Some carbohydrates have a high glycemic load (GL) that causes hyperinsulinemia postprandial, as a result, favouring the uptake of fatty acids, inhibiting lipolysis finally resulting in weight gain (Pittas *et al.*, 2005). Cereals, fibre and poly unsaturated fatty acids are recommended in the diabetic diet due to their low GL (Hu, 2011). Based on literature, consumption of sugar-sweetened beverages (SSBs) has a 26% risk of developing DM (Bray and Popkin, 2014). SSBs contain rapidly absorbable sugars, namely fructose and sucrose; these stimulate the beta pancreatic cells to release copious amounts of insulin possibly resulting in beta cell exhaustion (Bray and Popkin, 2014; Kahn and Sievenpiper, 2014).

On the other hand, bariatric surgery has been indicated as the best form of therapy in obese diabetic patients with body mass index (BMI) of ≥ 35kg/m2. It has been shown that 50-95% of patients who undergo bariatric surgery will eventually have near normal glycemic states. Other beneficial lifestyle changes include cessation of smoking and alcohol consumption (Hu, 2011).

2.8.2 Oral anti-diabetic drugs (OADs)

In view of the fact that diabetes exists in several different forms, unique combinations of OADs have been in place so as to maintain a normal glycemic status and thus prevent diabetic complications (Ohkubo *et al.*, 1995; Bastaki, 2005). Evaluations on the best algorithmic treatment have to be cautiously investigated. The best choice of pharmacological regimens should include the following properties, an effective glycemic control, minimal side effects, tolerable, cheap, readily available and mitigation of diabetic symptoms (Nathan *et al.*, 2009; Philis-Tsimikas, 2009). Pharmacological agents have been reported to exhibit adequate glycemic control despite their side effects (Nathan *et al.*, 2009). Medical practitioners worldwide are facing difficulties in decision making on the best algorithm to prescribe as well as when to initiate the therapy (Schmitz *et al.*, 2004; Nathan *et al.*, 2009). Timing on the pharmacological treatment is important as the delay in treatment results in

poor glycemic control in the long run potentiating the development of lethal complications (Jain and Saraf, 2010). The American Diabetic Association (2011) proposed that a < 7% HbA1c should be targeted to reduce diabetic complications.

2.8.3 Pharmacological drugs used in the treatment of DM

2.8.3.1 Metformin

Metformin (Met) is one of the OADs administered in the first line of treatment. The drug has anti-hyperglycemic effects and was derived from the *Galega officinalis* plant. Met is a member of the biguanide family, it acts by controlling hepatic glucose output achieved by reducing the rate of hepatic gluconeogenesis (Bailey, 2000). Metformin is safe in prediabetics because it has not been associated with hypoglycaemia and weight gain (Bailey 1992; Bailey, 2000).

2.8.3.2 Sulfonylureas

Sulfonylureas are drugs that cause hypoglycaemia by stimulating insulin secretion from pancreatic beta cells (Krentz and Bailey, 2005). Sulfonylureas act by binding to the sulfonylureas receptors found on β pancreatic cells' plasma membrane. The binding closes the ATP sensitive channel resulting in depolarisation of the membrane leading to the opening of the voltage channels allowing entry of calcium ions eventually causing insulin secretion (Akinmoladun *et al.*, 2014). Despite the rapid glucose lowering effect, sulfonylureas have been implicated in cardiovascular related mortality and excessive weight gain (Nathan *et al.*, 2009)

2.8.3.3 Thiazolidinediones

Thiazolidinediones (TZDs) can be referred to as glitazones. TZDs are peroxisome proliferator- activated receptor γ modulators (Nathan *et al.*, 2009). TZDs play a key role in increasing the peripheral sensitivity of muscles, liver and fat to insulin. However, the drug is associated with longer duration of hypoglycaemia. Oedema and weight gain are also major setbacks of administering TZDs (Bastaki, 2005).

2.8.3.4 Amylin

Amylin, also commercially known as pramlitide, is a synthetic analogue of human amylin. Human amylin is linked to amyloid deposition in the pancreatic tissue causing β cell destruction, hence the preference of pramlitide instead. Pramlitide demonstrates post prandial glucagon suppression, slowing of gastric emptying and carbohydrate absorption (Nathan *et al.*, 2009). The analogue can cause severe hypoglycaemia if used in conjunction with insulin (Schmitz *et al.*, 2004; Nathan *et al.*, 2009).

2.8.3.5 Alpha-glucosidase inhibitors

The α-glucosidase inhibitors are a group of drugs that decreases the rate of absorption of carbohydrates, thus lowering postprandial glucose concentrations. The major side effect that has been reported is gas production (Bolen *et al.*, 2007).

2.8.4 Medicinal Plants and their health benefits with focus on DM

Plants with medicinal values are known to play a significant role globally, but particularly in African and Asian regions in the management and treatment of various chronic diseases including diabetes. In African and Asian regions due to low socio-economic patterns, about 80% of these populations still rely greatly on plant products as their primary healthcare sources (Abo *et al.*, 2008; Akinmoladun *et al.*, 2014). The interest in medicinal plants has grown simply because they are readily available and they are linked to minimum side effects (Modak *et al.*, 2007). The relative cost of pharmacological drugs globally has raised concerns of diversion from synthetic drugs to phytotherapy. The cost of metformin has been reported to be almost 5 fold higher than that of the cheapest generic sulphonylureas, 6 fold higher for repaglinide and close to 30 fold higher for thiazolidinediones. The treatment and management cost of DM in developing countries is thus expensive, hence the predilection for phytotherapy (Vuksan and Sievenpiper, 2005).

It has been reported that more than 1000 plant species have been used to treat diabetes mellitus either as a single herb or as a combination of two or more herbs (Arise *et al.*, 2014). Therapeutic effects of plant extracts were linked to the presence of natural, raw and unprocessed medicinal constituents (Malviya *et al.,* 2010). Plant materials are rich in phytochemicals such as glycosides, alkaloids, terpenoids, flavonoids, beta glycans and carotenoids that possess bountiful medicinal effects (Eddouks *et al.*, 2002). Reports indicate that phytochemicals or bioactive constituents used in the treatment of DM provide

therapeutic effects either by combating reactive oxygen species (ROS) and or acting as hypoglycaemic agents (Jideani *et al.*, 2014). Various *in vivo* studies in animal models have shown the ability of specific plant material extracts to reinstate glucose levels to near normal levels (Shukla *et al.*, 2000; Ojewole, 2006; Ayepola *et al.*, 2013; Ayepola *et al.*, 2014; Oyenihi *et al.*, 2015). In the human body, different mechanisms work together to favour the pathogenesis of DM. Oxidative stress and free radical formation, as mentioned earlier, is the major trigger of DM complications as a result of non-enzymatic glycosylation, auto oxidative glycosylation and metabolic stress. To potentially prevent the onset of diabetic complications, diabetic oxidative stress can be reduced by consumption of plant materials rich in antioxidants (Sabu and Kuttan, 2002).

2.8.5 Possible mode of action of medicinal plants as anti-diabetic agents

Plants exhibit various anti-diabetic activities both *in vivo* and *in vitro.* Studies have shown that some plant extracts can inhibit the re-absorption of glucose in renal tissue thereby causing hypoglycaemia (Eddouks *et al.*, 2002). The inorganic component of plant materials consists mainly of essential minerals, namely calcium, zinc, potassium, manganese chromium etc. These minerals play a role in the release of insulin from beta pancreatic cells (Jideani *et al.*, 2014). Some plants components have inhibitory effects on the action of carbohydrate-degrading alpha glucosidase and beta galactosidase, resulting in decreased carbohydrate breakdown (Naowaboot *et al.*, 2008). It has been reported that while some plant components prevent bêta cell destruction and/ increase beta cell rejuvenation, others aid in the stimulation of glycogenesis and hepatic glycolysis (Miura *et al.*, 2001). Most plants' mode of action however, still remains unclear, hence the need for further studies to determine the exact mechanism of action (Jouad *et al.*, 2001).

2.9 Examples of some medicinal plants used in the treatment of DM in South Africa

The following plants have been reported to treat DM and are mainly used in the Eastern Cape region of South Africa. They include: *Artemisia afra jacq, Vernonia amygadalima Del, Bulbine frutescens, Apocynaceae catharanthus roseus* (Erasto *et al.*, 2005).

2.9.1 *Artemisia afra jacq*

Figure 2.5: *Artemisia afra jacq* **(Hillard, 1997)**

Artemisia afra jacq is a medicinal plant frequently used in South Africa to treat DM. *Artemisia afra jacq* is a shrub that usually reaches 2m in height with hairy leafy stems (Figure 2.5). Study done in 2011 by Afolayan and Sunmonu showed the plant's ability to reverse diabetic oxidative stress in the pancreas of STZ-induced diabetic rats due to the reduction of lipid peroxidation that was accompanied by an increase in the activity of antioxidant enzymes. The plant has been studied by Sunmonu and Afolayan (2013) again to substantiate its antidiabetic effects. In their 15 day experimental study, aqueous extracts (50, 100 and 200mg/kg) of *A. afra jacq* were fed to 3 groups of adult male Wistar rats. Two groups were STZ- induced but one was treated with plant extract while the other was just given distilled water. The effect of the plant on blood glucose level showed a significant reduction, with the greatest reduction observed from the 200mg/kg concentration. The oral glucose tolerance test indicated an equivalent blood glucose reduction between the plant extract and glibenclamide. The authors concluded that *A. afra jacq* prevents the progression of the symptoms of diabetes namely polydipsia and polyphagia.

2.9.1.1 *Bulbine frutescens L. Wild*

Figure 2.6 *Bulbine. frutescens* (Harris, 2003)

B. frutescens (Figure 2.6) is well known for its use as a topical ointment as well as a digestive tonic (Van Wyk, 2011). Apart from the above mentioned uses, the plant has been used as a hypoglycaemic agent in the treatment of diabetes. To support the claims, van Huyssteen and colleagues (2011) investigated *B. frutescens* effects in C2 C12 cell lines and Chang liver cells. Based on their findings, the plant extract improved glucose utility in these cell lines.

2.9.1.2 *Catharanthus roseus*

Figure 2.7: *Catharanthus. roseus* **plant (Robertson, 2008)**

C. roseus also known as *Vinca rosea* is a shrub with distinct purple or white flowers that has its origin in Madagascar (Figure 2.7) (van de Venter *et al.*, 2008). The hypoglycaemic nature of the plant was associated with the presence of various phytochemicals distributed throughout the plant. *C. roseus* was reported by Rasinenis *et al* (2010) to be an antihyperglycemic plant rather than a hypoglycaemic plant as it increases glycolysis and glucose oxidation (via the Shunt pathway). In a study, the authors (Rasinenis *et al.*, 2010) performed in diabetic rats, a 77.7% blood glucose reduction in treated diabetic rats was noted after 60 days of treatment. To validate the earlier findings, Singh and colleagues (2001) observed an increase in the activity of glucose metabolic enzymes as well as a decrease in lipid peroxidation as a driving factor in the development of diabetic complications. Increase in glucose utilisation in hepatocytes was reported by van de Venter *et al* (2008).

2.9.1.3 *Vernonia amygadalina*

Vernonia amygadalina, also known as the bitter leaf, grows wildly in the KwaZulu Natal, Mpumalanga and the Eastern and Northern Cape regions of South Africa (Afolayan and Sunmonu, 2010). In folk medicine, the plant is used to treat parasitic infections, diabetes, eczema and malaria. In attempts to confirm its anti-diabetic nature, few in vivo tests were carried out. *V. amygadalina* reverses diabetic oxidative stress (Nwanjo, 2005; Farombi and Owoeye, 2011). Observation by Nwanjo (2005) in a series of experiments in 3 groups of rats namely: normal control, STZ-induced non-treated and STZ-induced diabetic treated with 200mg/kg of the plant extract shows that malondialdehyde, glucose and lipid parameters measured in all groups indicated hypolipidemic, hypoglycaemic and antioxidant effects of *V. amygadalina* in diabetic treated group. The significant fall of triglyceride levels was associated with the delay of CVD progression. These results collaborated with the findings of Uadia (2003) in which a reduction of LDL, triglycerides and cholesterol was observed in diabetic Albino rats. A dose related reduction in blood glucose levels after *V. amygadalina* extract adminstration was reported by Akah and Okafor (2006) in adult alloxanised rabbits. In normal treated rabbits a fall from 96mg/kg-48mg/kg was observed while 520mg/kg-300mg/kg fall in alloxanised treated rabbits was reported (Akah and Okafor, 2006). The mode of action of this plant was suggested by Ebong *et al* (2003) to be associated with insulin production and correction of carbohydrate metabolism.

African potato plant

tuber/ corm

Cross section of the corm

After extraction, refinery and distillation

Figure 2.8: *Hypoxis hemerocallidea* **plant, (Drewes** *et al.*, **2008)**

Hypoxis is a family of plants that are extensively used for medicinal purposes in the Southern African region. This family consists of several types of species which include *H interjecta, H multicps, H nyasisa, H obtuse, H sobolifera* and the *H hemerocallidea* (Abegaz *et al.*, 1999). The Hypoxidaceae super family is made up of 8 genera and 130 species. Of the 130 species, more than 50% are found in Southern Africa (van Wyk *et al.*, 2000).

Taxonomically, *Hypoxis hemerocallidea* belongs to the hypoxidaceae (Star lily family) as mentioned above. This plant was first described by Linnaeus in 1759. The name was derived from the Greek words *hypo* (below) and *oxy* (meaning sharp), in reference to the ovary which is pointed at the base. The plant has recently drawn attention of researchers worldwide because of its beneficial medicinal effects. Geographically, the plant is mostly distributed in the Southern hemisphere and is mostly abundant in Southern Africa (Owira and Ojewole, 2009). In South Africa, the African potato wildly grows in the Eastern Cape and all the way up to the KwaZulu Natal area. It is also native in the savannah areas of Lesotho, Mozambique, and Zimbabwe and in the mountainous regions of South America, Australia and Asia (Drewes, 2008; Afolayan and Sunmonu, 2010).

H. hemerocallidea is commercially known as the African potato and has been also referred to as the miracle plant (Van Wyk, 2011). In South African vernacular, the plant is referred to as Ilabatheka, inkomfe or igudu (IsiZulu), sterretje/ afrikaan patat (Afrikans), inongwe (isiXhosa), tshuka (seTswana), molikharatsa (seSotho), star lily and yellow star in English (Singh, 1999; Erasto *et al.,* 2005; Mills *et al.*, 2005; Drewes *et al.,* 2008; Van Wyk, 2011). The plant is characterised by strap shaped leaves held on thick green hairy stems that are un-branched. The stems hold stalks supporting 2-12 yellow, star shaped flowers (Figure 2.8). At the base of the plant (just above the ground) lies the dark brown to black corm - a tuberous rhizome root stalk (10-15cm in diameter), that is erroneously referred to as the potato for this reason the plant is confused with the *Plectrantus esculentus* which is also known as the African potato (Figure 2.8) (Ojewole, 2006; Katerere and Eloff, 2008; Drewes *et al.*, 2008 Owira and Ojewole, 2009; Afolayan and Sunmonu 2010).

The tuberous part of the *H. hemerocallidea* is the one that is believed to possess bioactive compounds (Ojewole, 2006). In folk medicine, the African potato had been used for centuries to treat a catalogue of ailments that includes the following: arthritis, cancer, diabetes mellitus, high blood pressure, psoriasis, gastric ulcers, wounds, tuberculosis, urinary tract infections, cancer, asthma, central nervous system disorders, palpitations, cold, flue, headaches, prostate cancer, herpes simplex virus, burns, diahorea and epilepsy (Steenkamp, 2003;

Steenkamp *et al.,* 2006; Ojewole, 2006; Nair *et al.,* 2007; van Wyk, 2000, Drewes *et al.,* 2008; Afolayan and Sunmonu, 2010). Most of these reports were relatively anecdotal claims from traditional healers, however scientific studies confirmed that *H hemerocallidea* possess anti-diabetic, anti-inflammatory, antioxidant, anti-nociceptive, antibacterial, anticancer and anti-diarrhoeal effects (Drewes *et al.,* 2008).

Previous reports highlighted that the African potato has the constituents that boost immune status in HIV infected patients, contributing specifically by activities of β- sitosterol that modulates and boosts the immune systems by stabilising the CD4 lymphocytes. Nevertheless, the plant was grossly linked to the suppression of the bone marrow. For this reason, the use of the plant in HIV infected patients has been terminated in South Africa (Erlwanger and Cooper, 2008).

The therapeutic effects of the African potato was attributed to the presence of sterols, starols, sterolins, norlignan, daucosterols and hypoxide (Drewes and Khan, 2004; Nair *et al.,* 2007). Among these phytochemicals, daucosterols, beta sitosterol and hypoxide are mostly associated with therapeutic activities (Boukes *et al.,* 2008). Hypoxide is the major component isolated from the corm. Hypoxide ((E)-1, 5 bis-(4'-β D- glucopyranosyloxy-3'- hydroxyphenyl) pent-4-ene-yne) is a glycosylated norlignan that is derived from cinamic acid (Figure 2.9). In its natural form, hypoxide is inactive but can be hydrolysed to rooperol by the action of β glucosidase enzyme. This conversion occurs in the gastrointestinal system particularly in the large intestines in humans and in animals, bacterial β glucosidase catalyses the conversion. Rooperol is a dicatechol aglucone bioactive compound that has powerful antioxidant properties in human blood (Laporta and Funes, 2007).

Compound		R_1	R_2	R_3	R_4
1	Hypoxoside:	-OH	-O-β-D-glucopyranosyl	-OH	-O-β-D-glucopyranoz
2	Dehydroxyhypoxoside:	-H	-O-β-D-glucopyranosyl	-OH	-O-β-D-glucopyranosyl
3	Bis-dehydroxyhypoxoside:	-H	-O-β-D-glucopyranosyl	-H	-O-β-D-glucopyranosyl
4	Rooperol:	-OH	-OH	-OH	-OH
5	Dehydroxyrooperol	-H	-OH	-OH	-OH
6	Bis-dehydroxyrooperol	-H	-OH	-H	-OH
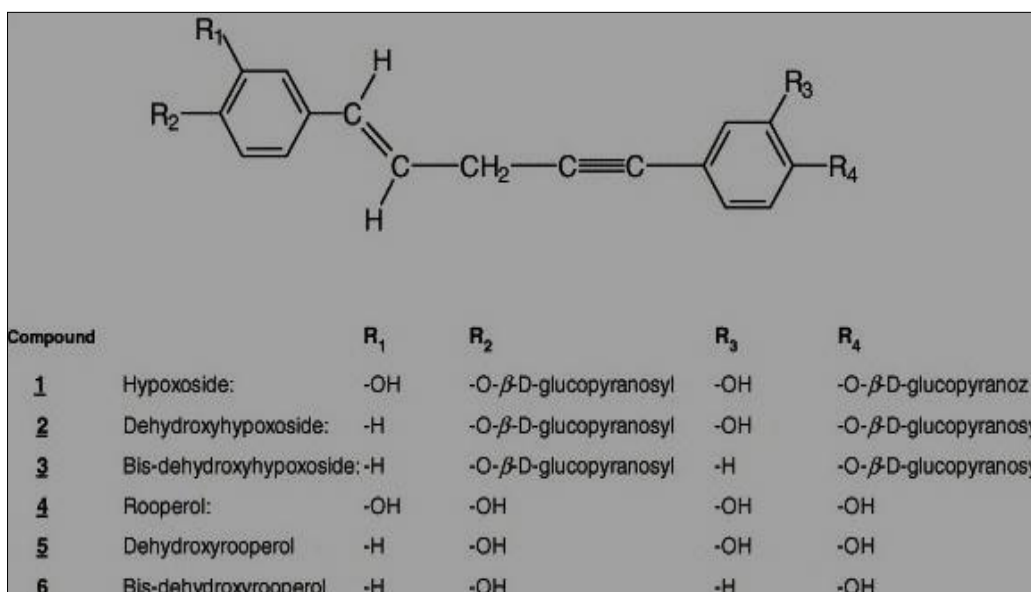
Figure 2.9: Chemical structures of norlignans derived from hypoxide (1-6)
         (Adapted from Larporta et *al.*, 2007)

2.9.2   Antidiabetic activities of *H. hemerocallidea* in experimental studies

With reference to the experimental studies done by Ojewole's group in 2006, the *H. hemerocallidea* was shown to demonstrate antidiabetic effects. The group administered streptozotocin (STZ) diabetic-induced rats with different concentrations of aqueous plant extracts (400-800mg/kg) had demonstrated antidiabetic effects. The extracts significantly reduced blood glucose levels in both fasted normal and non-fasted treated rats, though the mechanism of the hypoglycaemic effect was unknown. The presence of phytosterols was however linked to the hypoglycaemic action to a lesser extent. The authors explained that since they used an aqueous solution in the extraction process, little phytochemicals were extracted but were still sufficient to cause hypoglycaemia. Ojewole (2006) reported that the plant extract caused a maximum of 30-50% glucose reduction.

The antioxidant capacity of rooperol has also been evaluated using TBARS, TEAC and FRAP assays and has been shown that the high antioxidant power of rooperol contributes immensely to its hypoglycaemic effects (Laporta *et al.,* 2007). The ability to inhibit lipid peroxidation was compared to the activity of green tea and rooperol caused higher inhibition (Laporta *et al.,* 2007). Rooperol has the ability to scavenge HO¨, hence reduce membrane lipo-oxidation (Nair *et al.*, 2007). Due to the above experimental results, the consumption of *H. hemerocallidea* can be rationalised in societies, in the treatment and management of type 2 DM.

Research Hypothesis
For this study, it is hypothesised that the extract of *H. hemerocallidea* will potentially reduce hyperglycemic state in streptozotocin-induced diabetic male Wistar rats over a 6 week period.

Chapter 3

RESEARCH DESIGN AND METHODOLOGY

3.1 Ethical clearance and animal care

Experiments were performed in sixty healthy adult male Wistar rats weighing between 210 to 240g. The animals were purchased from University of Stellenbosch's Department of Physiological Sciences (South Africa). Different groups of animals were housed under standard environmental conditions (23±1 ∘C, 55±5% humidity) and were exposed to free water access, standard animal diet called Standard rat chow (SRC) and *H. hemerocallidea* aqueous extract depending on the group. Each group of animals (12 animals per group) received the same amount of water and SRC daily. Animals were housed in stainless steel cages with surfaces made of movable plastic for easy daily cleaning and maintenance. The animal feeding was conducted over a period of 6 weeks.

The ethical approval was obtained from the Faculty of Health and Wellness Sciences Research Ethics Committee (REC) of the Cape Peninsula University of Technology. All animals used received humane care from trained personnel and were treated with respect according to the principles of Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health publication no. 80-23, revised 1978).

3.2 Experimental treatment

3.2.1   Diabetes mellitus induction

All the groups of animals were fasted overnight and body weights and blood glucose levels were measured after fasting. Type 2 DM was induced in experimental groups via a single intraperitoneal injection of streptozotocin (STZ) at a dose of 50mg/kg body weight as used by Ayepola *et al.*, 2014a. The STZ was dissolved in 0.1M cold sodium citrate buffer, pH 4.5. After 72 hr, fasting blood glucose levels of all the animals were measured from blood obtained from the rat tail vein. Experimental animals with blood glucose levels greater than 15 mmol/l were considered to be diabetic using the Accucheck Glucometer (Roche, Germany). Animals that failed to respond to the STZ shot were re-injected with the same

concentration of STZ. Treatment with *Hypoxis hemerocallidea* commenced on the fourth day after STZ injection, this day was regarded as the first day of treatment.

3.2.2 Dietary supplementation

All rats were fed with the SRC purchased from AQUANUTRO Company (Mamelsbury South Africa).

Rodent Breeder composition

Table 3.1: Composition of rat diets

Nutrient	Amount g/kg	
Protein	160	(Minimum)
Moisture	120	(Maximum)
Lipid	25	(Minimum)
Fibre	60	(Maximum)
Phosphorus	7	(Minimum)
Calcium	18	(Maximum)

3.2.3 *H. hemerocallidea* preparation

Powdered extract of the *Hypoxis hemerocallidea* corm kept in an aluminium foil sealable bag and stored in a -90 degrees Celcius refrigerator. The identification and authentication of the plant was done by a Botanist at Afriplex, Parrow, Cape Town, South Africa with voucher number CRMD02093) and the voucher kept at the herbarium. Different concentrations of the extracts were made up in 1 ml of distilled water daily (200mg/kg and 800mg/kg body weight). Experimental and control groups were gavaged with designated concentrations of the plant extract and distilled water respectively. Treatment of rats with aqueous plant extract was done daily from the fourth day up to day 41 of the 6 weeks period.

3.2.4   Experimental Design

Rats were divided randomly into five groups. Each group was made up of twelve animals (n=12). Body weights were measured whenever glucose readings were taken. Glucose concentrations were measured twice a week (Mondays and Thursdays) after overnight fasting.

Group 1- Normal control

This group consisted of normal rats that were not subjected to diabetic condition. The group was exposed to equal amounts of SRC and water as rats in other groups. The rats were gavaged daily with 1ml of distilled water for six weeks.
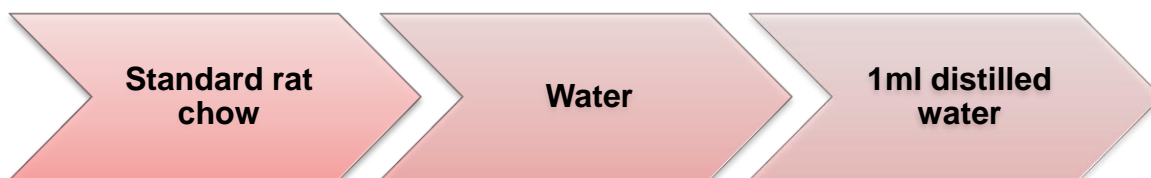
Figure 3.1: Experimental design for group 1

Group 2- Diabetic (STZ) controls

The 2nd group was the positive diabetic control group which received no treatment. The rats were fed with the same amount of SRC, water and treated with 1 ml of distilled water for six weeks.
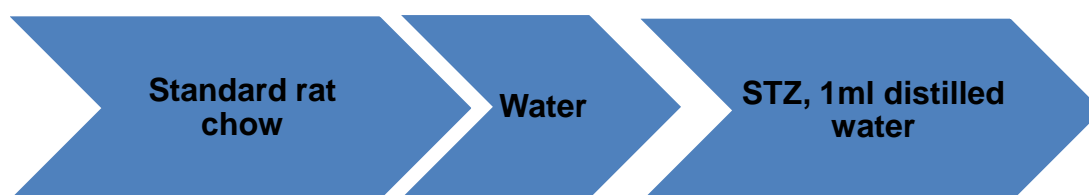
Figure 3.2: Experimental design for group 2 animals

Group 3- STZ + 800 mg/kg *H. hemerocallidea*

Rats were fed with the normal diet-SRC and water; however the group was treated with 800mg/kg body weight of the *H. hemerocallidea*. This group was a diabetic group in which each rat was injected with 1ml solution of the plant extract dissolved in distilled water for six weeks.
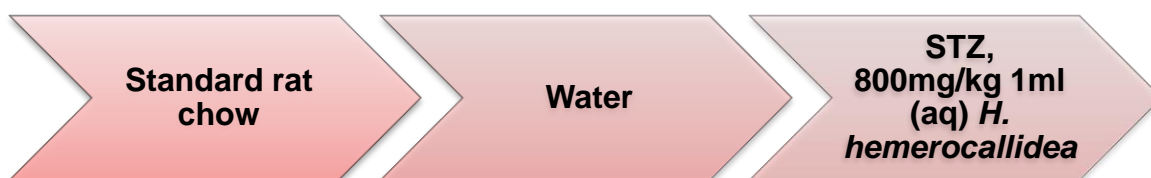
Figure 3.3: Experimental design for group 3 rats

Group 4- STZ + 200 mg/kg *H. hemerocallidea*

Diabetic group treated with 200 mg/kg of *H. hemerocallidea* made up in 1 ml of distilled water. Rats were subjected to normal diet (SRC and water), the treatment was done in six weeks.

Figure 3.4: Experimental design for group 4 animals

Group 5- Normal + 800 mg/kg *H. hemerocallidea*

Animals were normal rats (not injected with STZ), fed with SRC and free water supply. 800 mg/kg body weight of the aqueous plant extract was gavaged daily for 6 weeks.
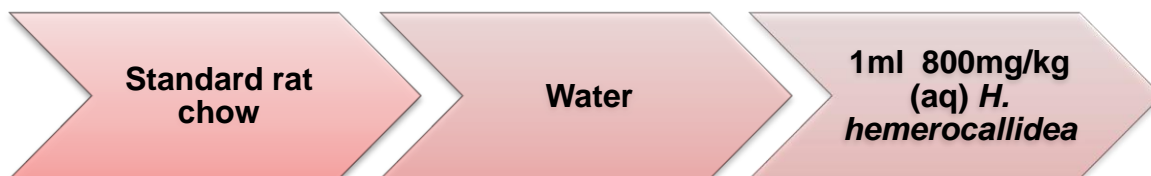
Figure 3.5: Experimental design for animals in Group 5

Abbreviations: *STZ: streptozotocin, aq: aqueous*

3.2.5   Blood and sample collection and preparation

On day 41 of the feeding period, the rats were fasted overnight in preparation for sacrifice and sample collection. Final blood glucose levels and body weights were recorded prior to sample collection. Rats were anaesthetised by the use of 1ml of 100mg/kg of sodium pentobarbitone; method of induction was via the intraperitoneal injection. After the rats were completely anaesthetised, blood samples were collected from the aorta using 5ml syringes connected to a 25 gauge hypodermic needles into 10ml serum separator vacutainer tubes (yellow top tubes). Tubes were allowed to stand at room temperature for 5-10 minutes before

being centrifuged at 3500 rpm for 15 minutes. Once centrifuged, the serum was transferred into cryo-tubes, frozen in liquid nitrogen and stored at -80 degrees Celsius.

Tissue samples (200mg liver and kidneys) of sacrificed animals were collected on ice, weighed, washed in phosphate buffered saline (PBS) to remove blood, and dried using a paper towel to remove excess PBS. The left side of the livers were minced into small pieces that were transferred into cryo-tubes, frozen in the liquid nitrogen and finally stored at -80 degrees Celsius for future analysis. The same procedure was also applied to the kidney samples. Stored tissue samples were homogenised in PBS mixed with 0. 5% triton X-100 at pH; 7.5. 200mg of tissue was added to 2000ul PBS and homogenised on ice. Homogenates were centrifuged at 4 degrees Celcius at a speed of 14000 rpm for 10 minutes. The supernatants were then transferred into labelled tubes and stored at -80° C.

3.3 Chemicals and materials

Tripyridyl-S-Triazine reagent (TPTZ), sodium reduced β NAD, glutathione reductase, hydroxydopamine hydrobromide, albumin reagent, creatinine reagent, urea reagent, total protein reagent purchased from RANDOX (South Africa). 2,2' azobisdihydrochloride (AAPH), 6- hydroxydopamine (6-HD), diethylenetriaminepentaacetic acid (DETAPAC), fluorescein sodium salt, ethylenediaminetetraacetic acid (EDTA), reduced β nicotinamide adenine dinucleotide phosphate (NADPH), 1-methyl-2vnyl-pyridinium trifluoromethane sulfonate, 5,5 Dithiobis- (2-nitrobenzoic acid) (DTNB), GSH standard solutions, glutathione reductase (GR), $FeCl_2$, metaphosphoric acid (MPA), perchloric acid (PCA), trichloric acetic acid (TCA), NA-Pi, 0,5% triton, 30% hydrogen peoxidesodium phosphate, potassium dihydrogen orthophosphate (KH_2 PO_4) 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), 6- hydroxyl-2,5,7,8-tetramethylchroman-2carboxylic acid (trolox), iron III chloride hexahydrate, L-Ascorbic acid were purchased from Sigma Aldrich (Johannesburg South Africa). Analytical pure grade solvents were used and these include sulphuric acid, gallic acid, hydrochloric acid (HCl), glacial acetic acid (Merk Johannesburg, South Africa).

3.4 Protein determination assay

The Thermo Scientific Pierce BCA protein assay kit was used for the colorimetric detection and quantitation of protein. The principle of this method is based on the reduction of Cu^{2+} to Cu^+ by proteins in alkaline conditions in the presence of bicinchoninic acid. The reaction is known as the biuret reaction where there is a reduction of the cupric ions which is noted by the purple coloration. Bovine serum albumin (BSA) was used as a standard where different concentrations were prepared by serial dilutions. The working reagent was prepared as per

procedure manual. In two micro plates, 25µl standard or sample homogenates in each well were mixed with 200µl of the working reagent and incubated at 37 $^{\circ}$C for 30 minutes. After cooling, the plates were read using the Multiskan plate reader at an absorbance of 562nm and results were expressed as mg protein/ml of tissue homogenates.

3.5 Assessment of total antioxidant capacity

3.5.1 Ferric Reducing Antioxidant Power (FRAP)

Ferric reducing antioxidant power is a colorimetric method that measures the ability of antioxidants to reduce oxidants. The method was developed in 1996 by Benzi and Strain. The method utilises Fe^{3+} ions as oxidants, the reducing power of the sample is determined by the conversion (reduction) of the ferric ion to the ferrous ion (Fe^{2+}). Antioxidants donate electrons to the ferric ions thereby reducing them; however this method does not depend on the concentration of antioxidants. The reaction occurs at low pH where the Fe^{3+} found in the (TPTZ) complex is reduced by antioxidants in a sample to the ferrous form (tripyridyltriazine complex) which is indicated by a blue colouration. Changes in absorbance are determined using a spectrophotometer at a wavelength of 593nm (Benzie and Strain, 1996; Molan *et al.*, 2008).

To determine the ferric reducing antioxidant power of tissue samples, the FRAP reagent was prepared by combining 30 ml of (300mM) acetate buffer at a pH of 3.6, 3ml of TPTZ solution, 3ml of $FeCl_3$ solution and 6ml of distilled water. L- Ascorbic acid solution (1mM solution, 0088g of Ascorbic acid + 50ml distilled water) was used as the standard and was used as a stock solution to prepare different concentrations of standards. 10µl of sample/ standard were pipetted into the 96 well plates, each sample was triplicated. 300µl of the FRAP reagent was added giving a total volume of 310µl in each well. The mixture was incubated at 37°C for 30 minutes. After the incubation period, readings were taken and results were compared to a standard curve that uses an equation (y= a + bx). Results were expressed as µmol AAEg^{-1} for both tissues.

3.5.2 The Oxygen Radical Absorbance Capacity Assay (ORAC)

ORAC is an assay that measures the ability of antioxidants in a particular sample to scavenge radicals. The principle of the assay is based on the scavenging and inhibition capacity of lipophilic antioxidants in the presence of a cyclodextrin water based enhancer upon the free radical damages. The free radical damage is evaluated by the loss of fluorescence of a fluorescent probe over time; therefore the decrease of fluorescence signifies extent of free radical damage and as well as a direct proportional relationship with

the free radical concentration. Decrease in fluorescence of the probe is therefore related to the weak scavenging activity of an antioxidant. Moreover, the longer it takes for the probe to lose its fluorescence, the stronger the scavenging and inhibition effects of the present antioxidant.

The 2- amino-propane dihydrochloride (AAPH) reagent is a source of the peroxyl radical used in the assay. Trolox solution (6-hydrox-2, 5, 7, 8-tetramethlychroman-2carboxylic acid) was used in the preparation of standards; the solution was prepared by mixing 0.00312g of trolox with 50ml phosphate buffer/ ORAC buffer (75mM, pH 7.4). The fluorescein was prepared by combining 0.0225g of the fluorescein sodium salt with 50ml ORAC buffer. 12µl of sample/ standards/ controls was added in well in triplicates, 138µl of the fluorescein was then added to each well of a black 96 microwell plate. 50µl of the AAPH was added last into the wells giving a final of 200µ. Fluorescence readings were measured by a fluoroskan ascent plate reader at 485nm excitation and 538nm emission wavelengths. The fluroskan took reading at the end of every 60 seconds for 2 hours. The scavenging effects of antioxidants were calculated by comparing the areas under the fluorescence curves of samples against the areas under curves of the controls. The regression equation ($Y=a+bX+cX^2$) was used to determine the ORAC values where Y= Trolox concentration in µM and X= net area under the fluorescence decay curve. Results were reported in units called trolox equivalents (TE) per millilitre/ µmol. The area under curve AUC was calculated as:

$AUC= (0, 5+f_2/ f_1 +f_3/ f_1 +f_4/ f_1 +………+ f_i/ f_{1)} \times CT$

Where f_1 is the initial reading at cycle 1, f_i is the reading at cycle I and CT is the cycle time in minutes.

3.6 Antioxidant enzymes

3.6.1   Catalase assay

Catalase is a biological antioxidant enzyme that converts reactive hydrogen peroxide molecules to non-reactive water and oxygen molecules. The catalase activity was determined spectrophotometrically in tissue homogenates according to the modified method of Ellerby and Bredesen (2000). Homogenates were prepared by measuring 200mg of tissue homogenised in 2ml of phosphate buffer on ice. Homogenates were centrifuged at 14000 rpm at 4 ^{o}C for 10 minutes. The catalase buffer was prepared by making a 50Mm (pH 7.0) of potassium phosphate whereas the hydrogen peroxide stock solution was prepared mixing

10ml of the catalase buffer with 34μl of 30% (v/v) hydrogen peroxide. 10μl of the homogenates was pipetted into micro plate wells first followed by 170μ l of phosphate buffer and 75μl of hydrogen peroxide lastly. The activity was evaluated using the Multiskan plate reader. Activity was calculated as follows:

Activity = [(A1-A2)/ Ę] × 0, 5/ μg protein = μmole / min /μg

Where Ę= 0, 00394 $Mm^{-1} .cm^{-1}$

3.6.2   Superoxide dismutase (SOD) spectrophotometric evaluation

Superoxide dismutase (SOD) activity was assessed by the use of the modified method of Ellerby and Bredesen (2000). 200mg of tissue were homogenised in phosphate buffer (50mM Na-Pi, 0,5% triton X -100) at a pH of 7,4. The SOD buffer was prepared from 50Mm $NaPO_4^-$ at a pH of 7, 4. 6- hydroxyl-dopamine solution was prepared by adding 20mg of 6-HD to 50ml of milliQ water and perchloric acid solution. Diethylenetriaminepentaacetic acid (DETAPAC) at a concentration of 0,1mM was prepared by adding 0,4mg in 10ml of SOD buffer. 12μl of the tissue homogenates were added in duplicates into the wells followed by 15μl) of the 6-HD solution and lastly 170μl of DETAPAC. The measurements were read at a 490nm wavelength at a 1 minute interval for 4 minutes. SOD activity was assessed from the linear calibration curve. The results were expressed in U/ml.

3.6.3   Measurement of total glutathione (GSHt) concentration

The glutathione redox analysis was determined by the method of Asensi *et al* (1999). The buffer (buffer A) was prepared from 500mM $NaPO_4^-$ in 1mM EDTA at a pH of 7,5, 1mM of NADPH was added to the buffer. 60mg of dithiobis-(2nitrobenzoic acid) (DTNB) at a concentration of 0,3mM was added to 500ml of buffer. Different standard concentrations were prepared by mixing buffer A with the standard stock solution. Gluthatione reductase solution was prepared by diluting GR with buffer A (80μl) of GR in 4920ul of buffer A). For GSSG analysis, tissues were homogenised in perchloric acid (PCA) 6% v/v that was mixed with 3Mm M2VP and 1mM EDTA solution. Tissues were homogenised in 15% trichloric acid with 1mM EDTA and were used for GSH analysis. 50μl of standards and samples were pipetted in triplicates in a 96- well plate, 50μl of DTNB and GR were added to each well using a multichannel pipette. The mixtures was incubated for 5 minutes at 25^oC, lastly 50μl of NADPH were added to each well to start the reaction, absorbance were measured at a wavelength of 412nm for 5 minutes. The GSH or GSSG program was selected depending on

the enzyme analysed. The linear slopes of standards were used to calculate the concentrations following the method below and was expressed as µmol/g and µmol/mg:

GSHt = µM × dilution factor (GSH = GSHt- 2GSSG)

GSSG = µM × dilution factor

Ratio = (GSHt − 2GSSG)/GSSG) = GSH

3.7 Analysis of total protein, albumin, creatinine, AST, ALT, ALP and globulin

All the biochemical, liver and kidney function tests were measured using the automated RANDOX chemistry analyser. All procedures were followed as according to the manufacturer's guidelines. The total protein was determined through a reaction that occurs between protein and cupric ions in alkaline environment. The serum albumin was measured based on its ability to bind to 3, 3', 5, 5'-tetrabromo-m cresol sulphonphthalein also known as bromocresol green. The complex formed was measured at 578nm. Globulin levels were calculated using the following equation:

Globulin = total protein - albumin

The creatinine levels were measured through a colorimetric method where creatinine reacts with picrate to form a coloured complex.

The serum activities of AST, ALT and ALP were measured to determine hepatic integrity. The principle of AST measurement is based on the reaction of α-oxoglutarate with L-aspartate in the presence of AST to form two products; L-glutamate and oxaloacetate. The oxaloacetate formed will further react with NADH to form L-malate and NAD$^+$.

$$\text{α-oxoglutarate + L-aspartate} \xrightarrow{\text{AST}} \text{L- glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate + NADH + H}^+ \xrightarrow{\text{MDH}} \text{L- glutamate + NAD+}$$
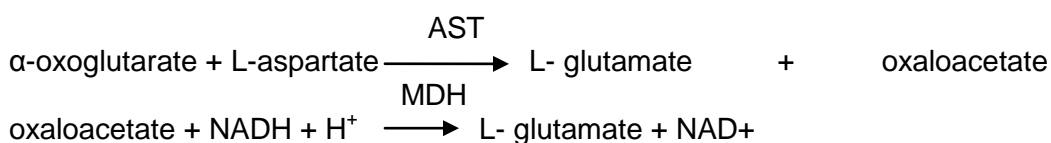
ALT activities are determined through the reaction of L-alanine and α-oxoglutarate in the presence of ALT resulting in the formation of L-glutamate and pyruvate. The consumption of NADH by pyruvate is used as a kinetic indicator. ALP measurements are used in the diagnosis of hepatobiliary, parathyroid, intestinal and bone diseases. The reaction that determines ALP utilises p-nitrophenyl phosphate as the substrate which is hydrolysed by ALP to form p-nitrophenol that is yellow in colour in the presence of magnesium ions. The amount of ALP present in the sample is directly proportional to the intensity of the colour.

3.8 Statistical analysis

The data were expressed as mean± standard error of the mean (SEM) and SD (standard deviation). Significant differences were analysed using the one way analysis of variance (ANOVA), the Bonferroni Multiple Comparison analysis was used to compare differences among groups by the use of the GraphPad ^{TM} PRISM5 software. At the P value < 0.05 the differences were considered to be significant

Chapter 4

RESULTS

4.1 Effects of the *H. hemerocallidea* plant extract on glucose levels

Figure 4.1 indicates glucose levels of rats in the different groups after diabetes induction using STZ and following *H. hemerocallidea* corm *(aq)* extract administration. Initial glucose readings show baseline glucose levels before induction of diabetes. Glucose readings after STZ induction represents glucose levels obtained four days after STZ administration and final glucose readings represent the glucose reading taken on day 41 of the experiment. After STZ induction, the fasting glucose levels increased by approximately 5 times the initial glucose readings and thus confirmed hyperglycemia. There was a significant change in glucose levels between the readings after STZ induction and the final glucose readings of group 3 following the administration of the plant extract (from 25.27 mmol/l to 6.74 mmol/l) and group 4 (from 24.53 mmol/l to 10.17 mmol/l) at *P< 0.05*. The greatest change was observed in group 3 and group 4 rats which had a 73.3% and 58.54% change respectively as indicated in Table 4.1. There were no significant changes in glucose levels observed in groups 1, 2 and 5 at *P<0.05* with 1.47%, 1.62% and 1.89% change respectively.
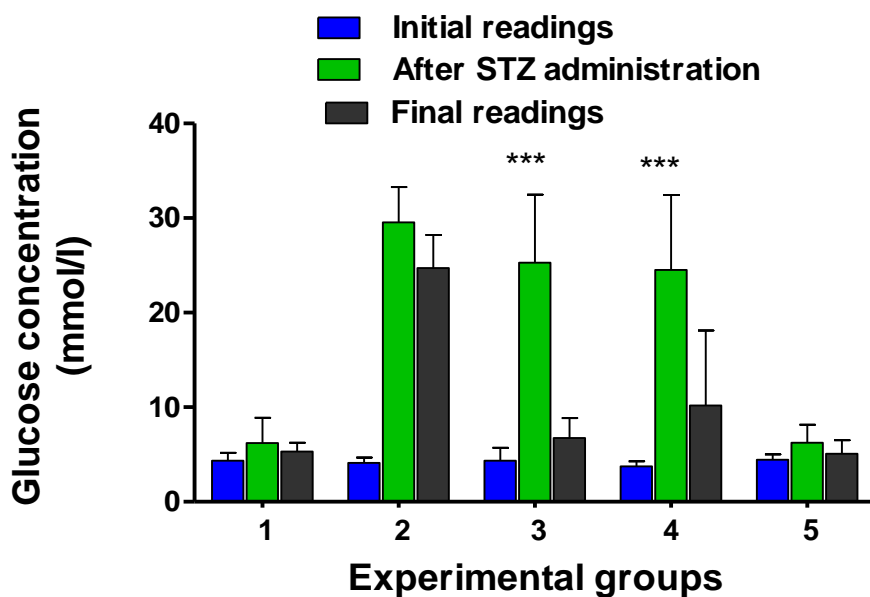
Figure 4.1: The effect of *H. hemerocallidea* on glucose concentrations

Abbreviations: mmol/l: milli moles per litre; *** represents significant difference at *P< 0.05* between the glucose readings after STZ administration and final glucose readings of respective groups.

Table 4.1: The percentage change of blood glucose levels following STZ induction and treatment with the H. hemerocallidea (aq) extract

GroupS	Glucose reading after STZ induction (mmol/L)	Final glucose reading after 6 weeks (mmol/L)	Percentage change (%)
Group 1	6.23	5.31	1.47
Group 2	29.54	24.73	1.62
Group 3	25.27	6.74	73.3
Group 4	24.53	10.17	58.54
Group 5	6.25	5.07	1.89

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.2 Effects of *H. hemerocallidea* extract on body weight

Figure 4.2 represent the effects of *H. hemerocallidea* (aq) extract on the body weights of STZ induced rats and normal rats. It can be noted that the initial body weights of all animals in all the groups ranged between 208g-217g. After the 6 weeks feeding period, significant increase in body weights between the initial and final weights at *P< 0.05* was observed in groups 1(normal untreated) and 5 (treated) controls where the final weights were 303.75g ± 23.68g and 302.9g ± 25.11g respectively. Administration of *H. hemerocallidea* extract in diabetic induced group 3 and group 4 did not significantly alter the final body weights in which the initial and final body weights for each group were (208.9g versus 229.2g ± 44.90g) and (210.917g versus 231.2g ± 35.59g) respectively. In addition, the diabetic control of group 2 showed no significant changes in body weight when comparing the initial and the final body weights at *P < 0.05* (215g versus 214g ± 25.51g).
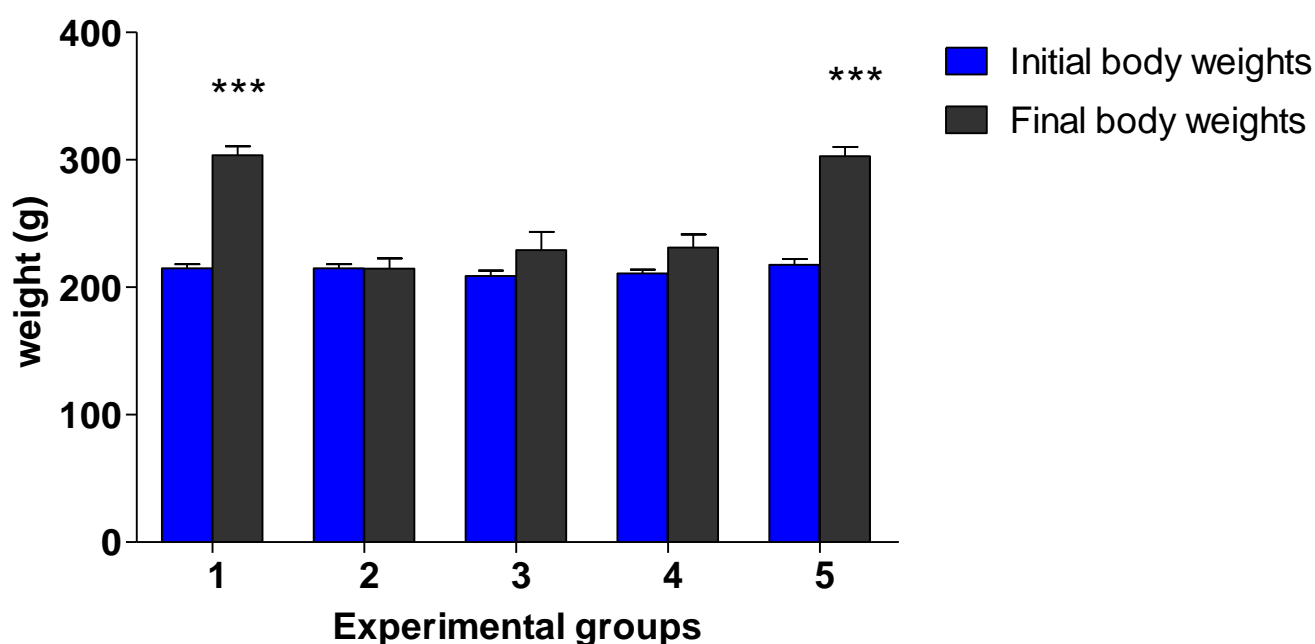
Figure 4.2: Effects of *H. hemerocallidea* extract on body weights

Abbreviations: g: weight in grams; *** represents significant difference at *P< 0.05* between the initial (before treatment) and final (after 6 week treatment period) weights of respective groups.

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.2.1   Effects of *H. hemerocallidea* on the final body weights

Figure 4.3 illustrates the effect of *H. hemerocallidea* (aq) extract on the final body weights of STZ induced diabetic and non-diabetic rats. A significant increase in body weights in group1 and group 5 was observed when compared to the diabetic non treated group 2, group 3 and group 4 at *P<0.05*. Treatment of diabetic induced rats (Group 3 and group 4) with *H. hemerocallidea* did not significantly increased body weights when compared to the normal untreated control group 1 at *P < 0.05* (229.2g ± 44.90g versus 303.75g ± 23.68g) and (231.2g ± 35.59g versus 303.75g ± 23.68g) repectively. There were no significant differences in the body weight of normal control group 5 treated with 800mg/kg when compared to the untreated normal control rats in group 1 (302.92g ± 25.11g versus 303.75g ± 23.68g). Furthermore, there were significant differences (lower body weights) in the final body weights of group 2, group 3 and group 4 when compared to the normal treated group 5 control (214.7g ± 25.51g versus 302.92g ± 25.11g; 229.2 ± 44.90 versus 302.92g ± 25.11g and 231.17 ± 35.59   versus 302.92g ± 25.11g at *P < 0.05*) respectively. Diabetic treated groups 3 and 4 showed no significant differences in final body weight when compared to the diabeti control (group 2) (229.2 ± 44.90 versus 214.7g ± 25.51g and 231.17 ± 35.59 versus 214.7g ± 25.51g at *P < 0.05*) respectively.
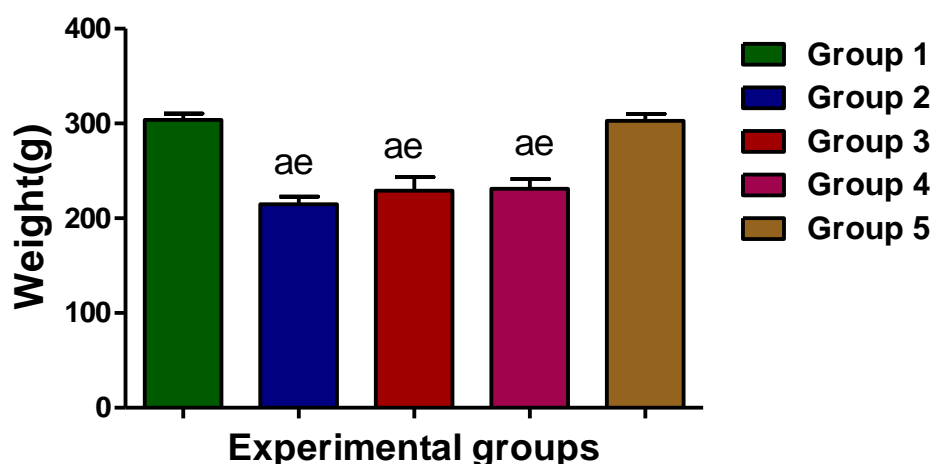
Figure 4.3: Effects of *H. hemerocallidea* on final body weights.

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*;

(e) indicates significant difference of groups when compared to group 5 at *P <0.05*.

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.2.2 Evaluation of liver weights of rats sudjected to diabetes and *H. hemerocallidea* treatments

Table 4.2 shows liver weights of rats administered with STZ at two different concentrations of *H. hemerocallidea.* The diabetic control group 2 showed significantly higher relative liver weights when compared to the normal control group 1 and the group 5 (4.72g versus 3.45g and 4.72g versus 3.34g $P< 0.05$). The diabetic group 3 treated with *H. hemerocallidea* also had significantly higher relative liver weights when compared the normal control group 1 and group 5 (4.31g versus 3.45g and 4.31g versus 3.34 at $P < 0.05$). However, the liver weights of diabetic rats treated with 200mg/kg of *H. hemerocallidea* did not show any significant difference when compared to group 1 and group 5 relative liver weights at $P < 0.05$. No significant difference in relative liver weights was observed in group 3 when compared to the diabetic control group 2 (4.31g versus 4.72g $P < 0.05$).

Table 4.2: Shows the relative liver weights after treatment with *H. hemerocallidea* in diabetic and non-diabetic groups

Group	Final Body weight(g)	Liver weight(g)	Relative(g/100g)
Group 1	303.75 ± 23.68	10.47±0.4196	3.45± 0.0014
Group 2	214.7± 25.51 [a,e]	10.15±0.3854	4.72± o.oo2[a]
Group 3	229.2 ± 44.90 [a,e]	9.89±0.5044	4.31± 0.0015[a]
Group 4	231.17 ± 35.59 [a,e]	9.11±0.33.13	3.94± 0.0021
Group 5	302.92 ± 25.11	10.13±0.2966	3.34± 0.001[b,c]

Abbreviations: (a)   Indicates significant difference when compared with control group 1 at $P < 0.05$

(b)   Indicates significant difference when compared with control group 2 at $P < 0.05$

(c)   Indicates significant difference when compared with group 3 at $P < 0.05$

(e)   Indicates significant difference when compared with control group 5 $P < 0.05$

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of H. hemerocallidea;

Group 4   : Diabetic group fed with 200mg/kg of H. hemerocallidea;

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of H.hemerocallide

4.2.3 Effects of *H. hemerocallidea* on the kidney weights

The Figure 4.4 shows the effect of H*. hemerocallidea* (aq) extract on the kidney weights of STZ diabetic induced rats. The kidney weights of the STZ untreated group and group 3 rats were significantly higher than those of the normal controls and group 5 rats at *P< 0.05*. There was no significant difference in kidney weights of rats in group 2 when compared to the kidney weights of rats in group 3 and group 4 at *P<0.05*. In addition, kidney weights in group 4 rats showed no significant difference when compared to the normal control and group 5 at *P < 0.05*.
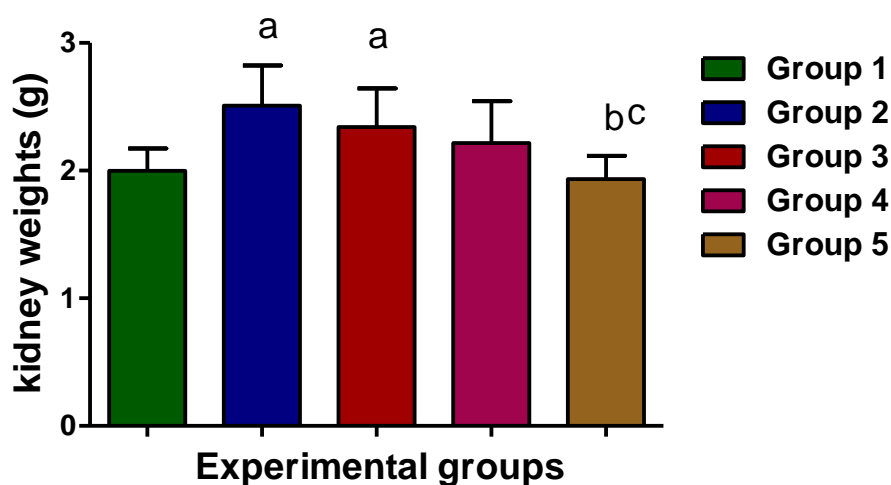
Figure 4.4: Effects of *H. hemerocallidea* on the kidney weights

Abbreviations: (a) Indicates significant difference when compared with control group 1 at *P< 0.05*
(b) Indicates significant difference when compared with control group 2 at *P< 0.05*
(c) Indicates significant difference when compared with control group 3 at *P< 0.05*

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of H. hemerocallidea;

Group 4   : Diabetic group fed with 200mg/kg of H. hemerocallidea;

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of H.hemerocallidea

4.2.4   Table 4.3 represents the effect of H*. hemerocallidea* (aq) extract on the relative weight of kidneys.

Table 4.3 represents the effect of *H. hemerocallidea* (aq) extract on the relative weight of kidneys of STZ induced and normal rats. The relative kidney weights of the STZ untreated group 2 were significantly higher than those of the normal controls (1.17g versus 0.66g) at P< 0.05. A significant difference in kidney weights of rats in group 2 and group 5 was noted where the weights in group 2 were elevated (1.17g versus 0.63g) at *P<0.05*. Rats treated with 200mg/kg also showed significant higher relative kidney weights when compared to the normal control group and group 5 rats at *P< 0.05.* No significant differences in relative kidney weights were observed when group 3 and group 4 rats were compared to the diabetic control group 2 at *P< 0.05*.

Table 4.3: Shows the relative kidney weights after treatment with *H. hemerocallidea* in diabetic and non diabetic groups

Group	Body weight(g)	Kidney weight(g)	Relative(g/100g)
Group 1	303.75± 23.68	1.997± 0.051	0.66± 0.0002
Group 2	214.7± 25.51	2.51± 0.099	1.17± 0.0005 [a]
Group 3	229.2± 44.90	2.341± 0.096	1.02± 0.001 [a]
Group 4	231.17± 35.59	2.218± 0.094	0.96± 0.002 [a]
Group 5	302.92± 25.11	1.933± 0.053	0.63± 0.001 [b c d]

Abbreviations: (a)   Indicates significant difference when compared with control group 1 at
                      P < 0.05
                   (b)   Indicates significant difference when compared with control group 2 at
                      P < 0.05
                   (c)   Indicates significant difference when compared with control group 3 at
                       P < 0.05

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of H. hemerocallidea;

Group 4   : Diabetic group fed with 200mg/kg of H. hemerocallidea;

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of H.hemerocallidea

4.3 Effects of *H. hemerocallidea* extract on total protein

The effects of *H. hemerocallidea* on serum total protein after 6 weeks experimental period are indicated by the graph in Figure 4.5. The graph shows significant reduced total protein values of STZ induced group 2, 3 and group 4 when compared to group 1. The normal treated group 5 did not show significant difference in the total protein when compared to group 1 (54.3636g/l ± 0.7883g/l versus 56.466g/l ± 0.7280g/l) at *P<0.05.* However, diabetic rats treated with 800mg/kg and 200mg/kg of *H. hemerocallidea* (aq) had significant lower serum total protein when compared to group 5 (50.31g/l ± 0.7295g/l versus 54.3636g/l ± 0.7883g/l and 51.18g/l ± 0.8280g/l versus 54.3636g/l ± 0.7883g/l) respectively at *P<0.05.*
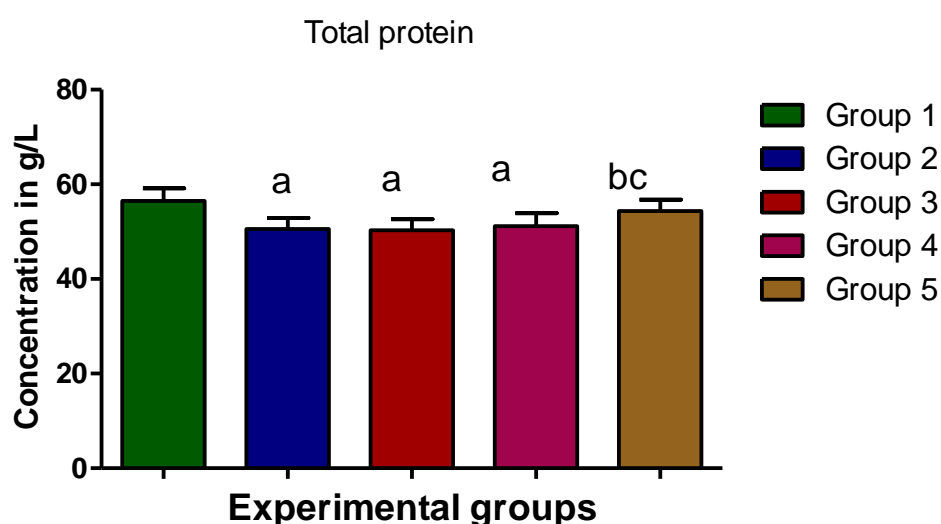
Figure 4.5 : The effects of *H. hemerocallidea* extract on total protein levels

Abbreviations: (a),(b),(c) Indicates significant difference when compared with control group 1, 2 and 3 respectively at *P < 0.05*

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.4 Effects of *H. hemerocallidea* on albumin values

Figure 4.6 shows the effects of *H.hemerocallidea* (aq) extract on serum albumin levels. After 6 weeks of feeding and treatment, there was significant decrease in serum albumin levels in groups 2, 3 and 4 at *P<0.05* when compared to the group 1 and group 5 controls. The serum albumin levels in group 3 and group 4 did not show significant differences when compared to serum albumin levels of the diabetic control rats at *P< 0.05*). Moreover, serum albumin levels of normal controls treated with 800mg/kg of the extract did not show significant differences to the serum albumin levels of normal controls at *P<0.05*.
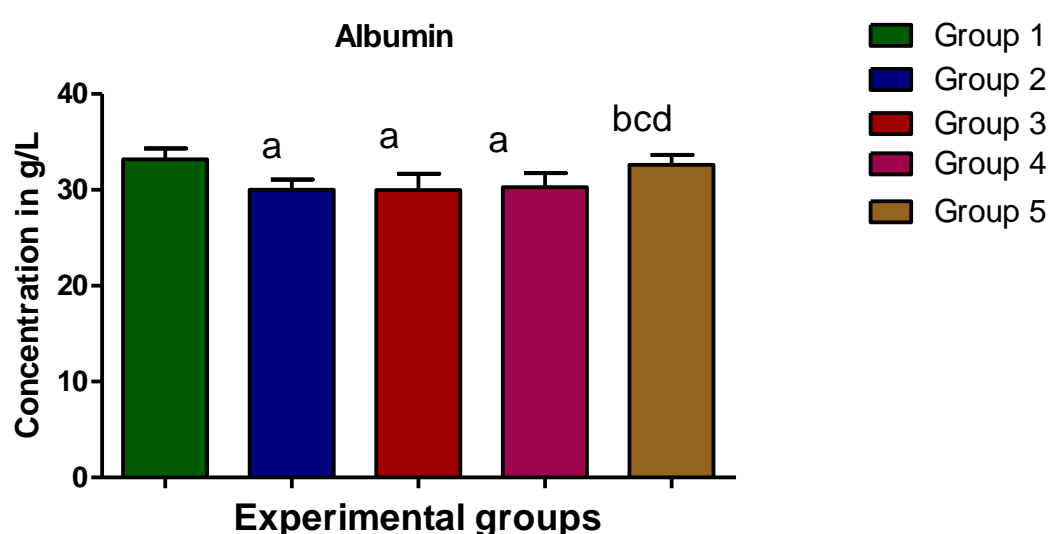
Figure 4.6: The effects of *H. hemerocallidea* aq extract on the albumin concentration

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents a significant differences of values in groups when compared to group 3; (d) indicates significant difference of groups when compared to group 4 at *P <0.05*; U/ug: units per micro gram

Experimental groups
Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.4.1 Effects of *H. hemerocallidea* on globulin levels

Table 4.2 indicates the results of *H. hemerocallidea* administration on globulin levels of STZ and non STZ induced diabetic rats. There was no significant change observed in globulin levels of all groups when compared to the normal control levels at $P < 0.05$.

Table 4.4: Effects of *H. hemerocallidea* on total protein, albumin and globulin serum levels

Group	Total Protein (g/L)	Albumin (g/L)	Globulin (g/L)
Group 1	56.4667 ± 0.7883	33.1933 ± 0.3289	23.2734
Group 2	50.5857 ± 1.873[a]	30.01 ± 0.3393[a]	20.5757
Group 3	50.31 ± 0.295[a]	20.998 ± 0.5380[a]	20.312
Group 4	51.1818 ± 0.8280[a]	30.28 ± 1.242[a]	20.9018
Group 5	54.3636 ± 0.7280[b c]	32.6073± 1.294[b c d]	21.7563

Value in columns indicate means and ± standard error means (SEM. (a) represents a significant difference when compared to group 1 control at *P <0.05*. There is no significant difference among groups for albumin and globulin levels. The globulin levels was calculated using the following equation:

Globulin = total protein – albumin (g/L)

Abbreviations: g/L: grams per litre

Experimental groups

Group 1   : Normal control;
Group 2   : Diabetic control;
Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*
Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*
Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.5 Effects of *H. hemerocallidea* on serum creatinine levels

Figure 4.7 represents the effects of *H. hemerocallidea* aqueous extract on the serum levels of creatinine. Serum creatinine levels in group 2 rats were the highest and there was a significant difference noted between group 1 and group 5 when compared to group 2 (68.45umol/l ± 2.133 umol/l versus 60.00 umol/l ± 1.036 umol/l) at *P < 0.05*. There was also a significant lower creatinine serum levels in group 3 when compared to group 2 (62.17umol/l ± 2.034umol/l versus 68.45umol/l ± 2.133 umol/l) at *P < 0.05*. The serum creatinine levels in group 4 animals were not significantly different when compared to group 1 controls. In addition, no significant differences in serum creatinine levels of group 5 animals were observed when compared to the normal control group 1 at *P < 0.05*.
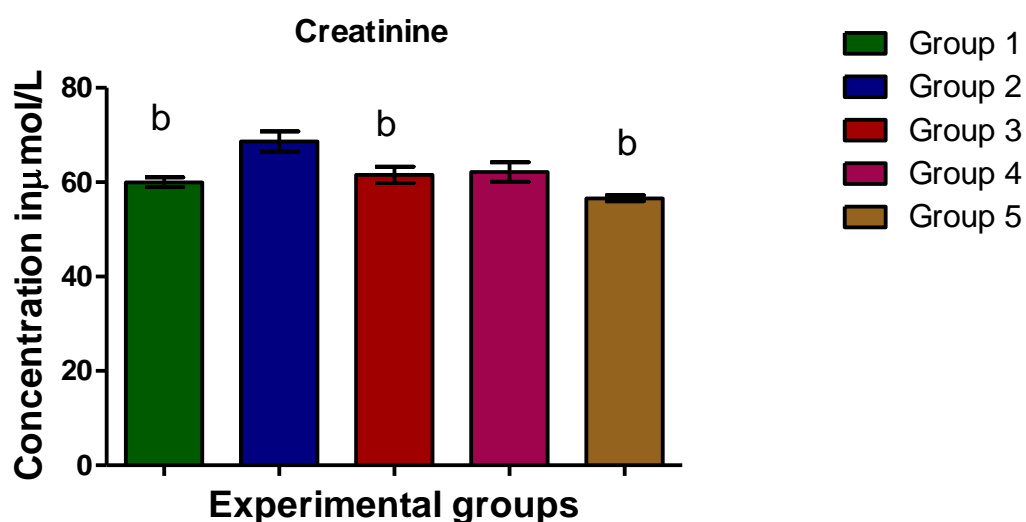
Figure 4.7: Effects of *H. hemerocallidea* on creatinine serum levels

> ***Abbreviations:*** (b) Indicates significant difference when compared with diabetic control group 2 at *P< 0.05*; **μ**mol/L: micro moles per litre

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.6 Ameliorative effects of *H. hemerocallidea* aqueous extract on liver function

The Table 4.5 represents the result of *H. hemerocallidea* (aq) extract on the activities of hepatic marker enzymes. The serum levels of all the enzymes in group 2 were significantly elevated when compared to the normal control group 1 and group 5 at *P < 0.05*. There was no significant difference in the activities of the enzymes in group 3 when compared to activities in group 2 at *P < 0.05*. Treatment with the 200mg/kg of *H. hemerocallidea* extract significantly reduced the serum levels of ALT and ALP when compared to the group 2, the diabetic group. Administration of *H. hemerocallidea* (800mg/kg) (aq) in normal controls did not result in significant changes in the serum levels of AST, ALT AND ALP when compared to the normal controls in group 1.

Table 4.5: Effects of *H. hemerocallidea* aqueous extracts on liver function.

Treatment groups	AST(U/L)	ALT (U/L)	ALP(U/L)
Group 1	142.2 ± 11.30	74.22 ± 7.865^{b}	136.545 ± 8.465
Group 2	235.5 ± 39.53^{a}	120.889 ± 8.559	537.889 ± 68.49^{a}
Group 3	237.67 ± 26.40^{a}	85.667 ± 8.077^{b}	424.222 ± 71.32^{a}
Group 4	183.636 ± 20	79.7778 ± 11.60^{b}	214.9 ± 50.95^{abc}
Group 5	179.455 ± 15.95^{c}	79.33 ± 6.754^{b}	134.917 ± 11.21^{bcd}

Value in columns indicate means and ± standard error means (SEM. (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents a significant differences of values in groups when compared to group 3.

Abbreviations: U/L: units per litre

Experimental groups

Group 1   : Normal control;
Group 2   : Diabetic control;
Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*
Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*
Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea*

4.7 The antioxidant effects of *H. hemerocallidea* on antioxidant statuses

4.7.1   The oxygen radical absorbance capacity (ORAC) of *H. hemerocallidea* in the liver

The effect of *H. hemerocallidea* on the oxygen radical absorbance capacity in the liver tissues is indicated in Figure 4.8. The ORAC values were significantly decreased in groups 2 and 4 when compared to the normal control group at *P<0.05*. There was a significant increase in the ORAC value in group 3 when compared to the ORAC values in groups 2 and 4 at *P< 0.05*. At *P < 0.05*, no significant changes were observed in groups 3 and 5 when compared to the normal control group 1. Group 2 diabetic controls also had significantly lower oxygen radical absorbance capacity when compared to group 4 rats fed with 200mg/kg of *H. hemerocallidea* (aq) extract at *P< 0.05*.
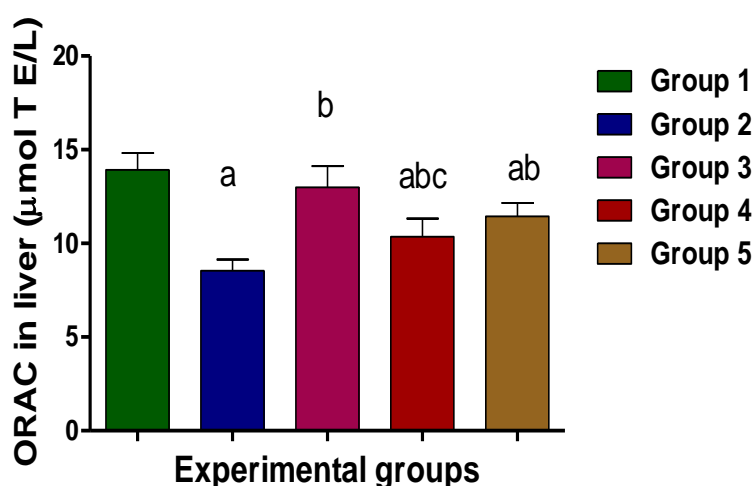
Figure 4.8: Figure 4.8 represents the effects of oxygen radical absorbance capacity of *H. hemerocallidea* in liver tissues

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*;

(b) indicates significant difference in groups when compared to group 2 at P <0.05 and (c) represents

a significant difference of values in groups when compared to group 3

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.7.2   The evaluation of the ferric ion reducing antioxidant power of *H. hemerocallidea* in the liver

The effects of *H. hemerocallidea* on the ferric ion reducing power in the liver tissue of normal and treated groups are indicated in Figure 4.9. The FRAP values were significantly higher in treated groups (groups 3, 4 and 5) when compared to the normal control group1 and the diabetic group 2 at *P< 0.05*. No significant changes were observed at *P < 0.05* in the FRAP value of the diabetic group 2 when compared to the normal control group. In addition no significant differences were observed when comparing group 3 amd group 4 FRAP values to the group 5 treated non-diabetic rats at *P< 0.05*.

Figure 4.9 : Effects of *H. hemerocallidea* extract on the FRAP in the liver tissue

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents a significant difference of values in groups when compared to group 3; μmol/g: micro moles per gram

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5 : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.8 Effects of H. hemerocallidea on endogenous antioxidant enzymes (SOD, catalase and GSHt)

4.8.1 Effects of *H. hemerocallidea* (aq) extract on the catalase activity in the liver tissue

The effect of *H. hemerocallidea* extract on catalase activity in the liver tissue is shown in Fig 4.10. Significant differences were noted among all the groups when compared to the diabetic normal control at *P<0.05* where there were significant increase in catalse activity in the normal control and all treated groups. There was no significant difference between group 3, 4 and 5 when compared to group 1 at *P< 0.05*.
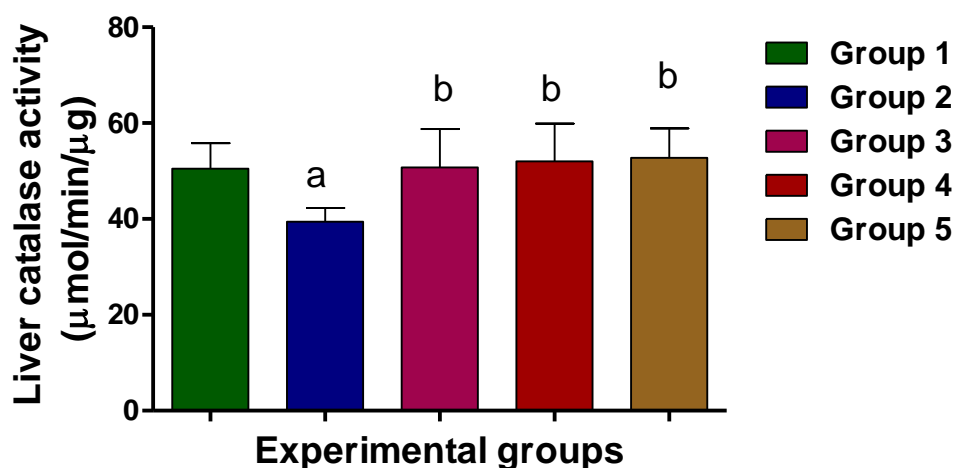
Figure 4.10: Effects of *H. hemerocallidea* on catalase activity in the liver tissue

Abbreviations: aq: aqueous; μmol/min/μg): micro mole per minute per micro gram; (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05*

Experimental groups
Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.8.2 Effects of *H. hemerocallidea* (aq) extract on the superoxide dismutase activity in the liver tissue

The effects *of H. hemerocallidea* extract on the SOD activities in the liver tissue are represented by Fig 4.11. No significant differences were noted in all the groups when were values compared to the normal control at *P<0.05*. Treatment of diabetic induced groups 3 and 4 showed no significant differences when activity was compared to the diabetic control group 2 at *P<0.05*.
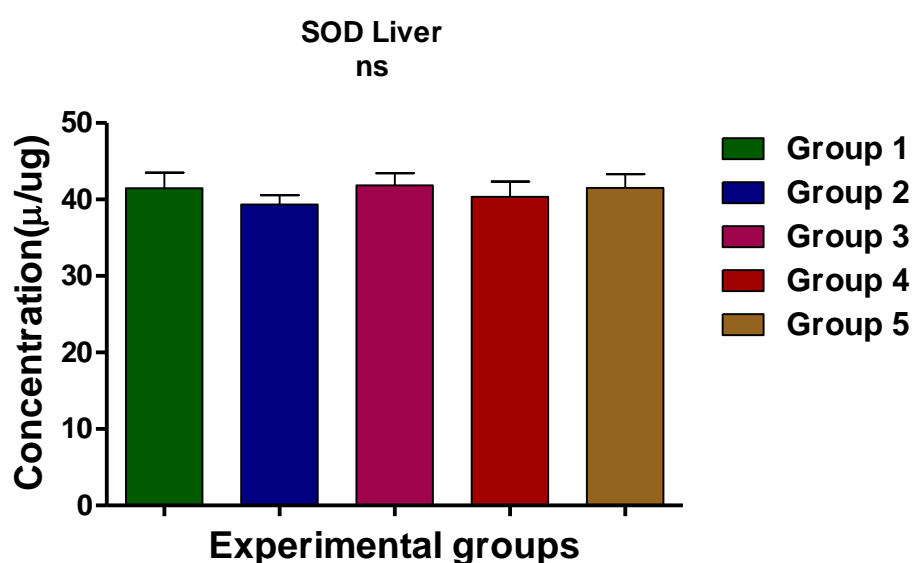
Figure 4.11: The effects of *H. hemerocallidea* on SOD activity in the liver

Abbreviations: ns: non significant difference between groups; U/:µg) nits in micro grams; aq: aqueous

Experimental groups

Group 1	: Normal control;
Group 2	: Diabetic control;
Group 3	: Diabetic group fed with 800mg/kg of *H. hemerocallidea;*
Group 4	: Diabetic group fed with 200mg/kg of *H. hemerocallidea;*
Group 5	: Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.8.3 Effects of *H. hemerocallidea* (aq) extract on the total GSH activity in the liver tissue

The effects of *H. hemerocallidea* aqueous extract on GSH activity in the liver tissues of normal and diabetic rats is represented in Figure 4.12. There were significant differences in the total glutathione activities in group 2 when compared to group 1 and group 5 at $P < 0.05$. The results show significant increases in GSHt activity in group 1 and group 5 when compared to the diabetic control group 2. Treatment of diabetic induced group 3 and 4 with the aqueous extract of *H. hemerocallidea* did not show significant differences in GHSt activity when compared to the group 1 and group 5 at $P < 0.05$.
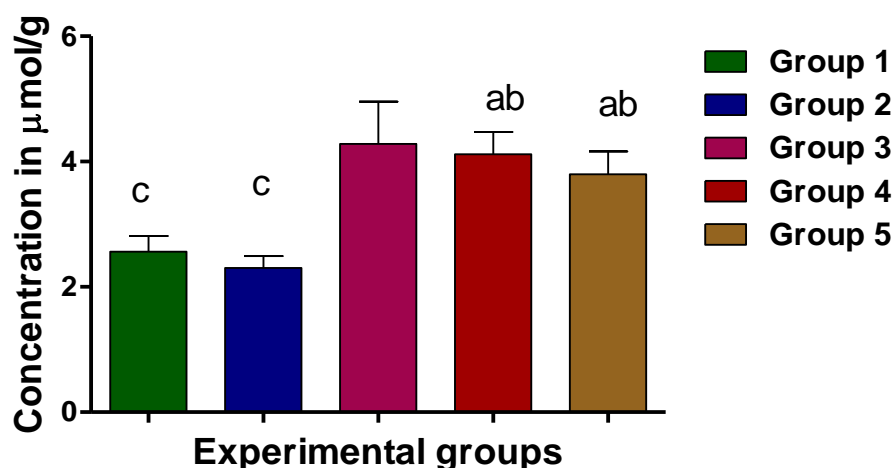

Figure 4.12: Effects of *H. hemerocallidea* (aq) extract on GSH activity in the liver tissue

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (e) indicates significant difference of groups when compared to group 5 at *P <0.05*
U/:μg) nits in micro grams; aq: aqueous

Experimental groups

Group 1 : Normal control;

Group 2 : Diabetic control;

Group 3 : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4 : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5 : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.9 The oxygen radical absorbance capacity of *H. hemerocallidea* aqueous extract on the kidneys

The effects of *H. hemerocallidea* on the oxygen radical absorbance capacity in the kidney tissues are represented in Figure 4.13. The ORAC values were significantly decreased in groups 2 and 3 when compared to the normal control group at *P < 0.05*. There was a significant increase in the ORAC value in group 5 when compared to the ORAC value in group 3 at *P < 0.05*. At *P < 0.05*, no significant changes were observed in groups 4 and 5 when compared to the normal control group 1.
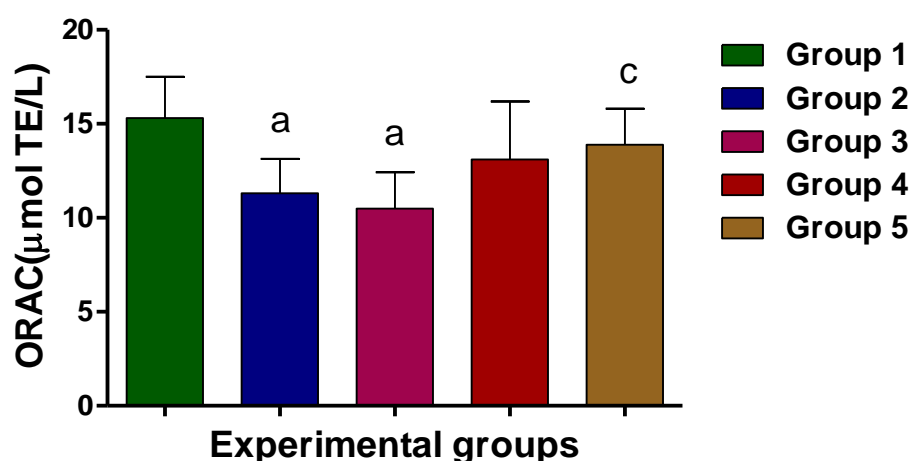
Figure 4.13: Figure 4.14 represents the oxygen radical absorbance capacity of *H. hemerocallidea* in kidney tisues

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (c) represents a significant difference of values in groups when compared to group 3 at *P <0.05*; μmol TE/L: micro moles trolox equivalents per litre.

Experimental groups
Group 1   : Normal control;
Group 2   : Diabetic control;
Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.9.1 The evaluation of the ferric ion reducing antioxidant power of *H. hemerocallidea* extract in the kidney tissue

The ferric ion reducing antioxidant power of *H. hemerocallidea* in the kidneys is shown in Fig 4.14. There were significant differences between group 4; group 5 and group 3 when compared to group 1 at *P< 0.05*. There was a significant decrease in the ferric ion reducing antioxidant power in group 4 and 5 when compared to groups 1 and group 2 at *P < 0.05*. No significant difference was noted in the FRAP value of group 2 when compared to the normal control FRAP value at *P< 0.05*. H. hemerocallidea (aq) extract administration in group 5 normal rats significantly reduced the ferric reducing antioxidant power when compared to group 3 animals at *P< 0.05.*
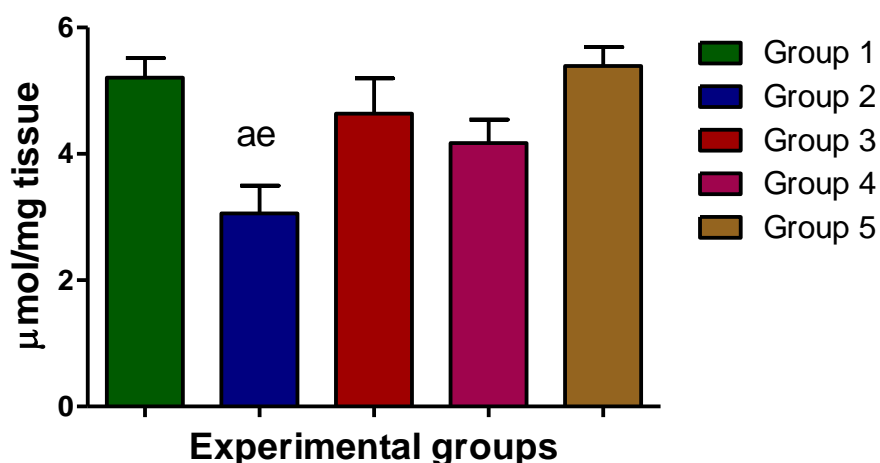

Figure 4.14: Effects of *H. hemerocallidea* extract on the FRAP in the kidney tissue

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents a significant difference of values in groups when compared to group 3 at *P <0.05* ; µmol/g: micro moles per gram

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea*

4.9.2   Effects of *H. hemerocallidea* (aq) extract on the catalase activity in the kidney tissue

The effects of *H. hemerocallidea* extract on the catalase activity in the kidney tissue are represented by Fig 4.15. There was a significant difference in catalase activity between group 2 and group 4; group 2 and group 5 and between group 3 and group 5 at *P<0.05*. There were no significant differences in catalase activities at *P<0.05* between group 1 and group 3; group 2 and group 3 when groups were compared. Furthermore, no significant differences were observed in group 5 catalase activity when compared to group 1 normal controls at *P< 0.05.*
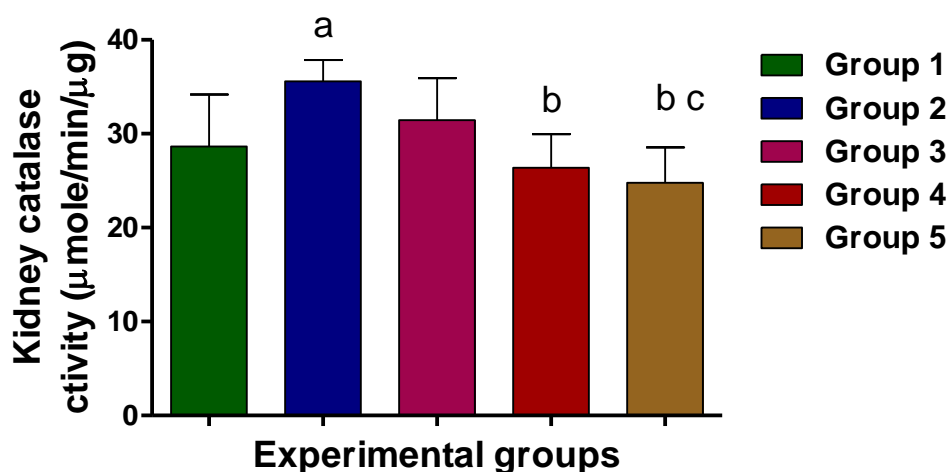
Figure 4.15: Effects of *H. hemerocallidea* on the catalase activity on the kidney tissue

Abbreviations: (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents significant differences of values in groups when compared to group 3 at *P <0.05*; μ mol/min/μg): micromoles per minute per micro gram of protein

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.9.3 Effects of *H. hemerocallidea* (aq) extract on the superoxide dismutase activity in the kidney tissue

Figure 4.16 indicates the effect of *H. hemerocallidea* extract on the activity of SOD in the kidneys of STZ induced and normal rats. Administration of *H. hemerocallidea* extract in normal rats viz: group 5 produced a significant change in the activity of SOD when compared to all groups at *P < 0.05*. There were no significant changes observed between groups 2, 3 and 4 when activities were compared to that of the normal control at *P < 0.05*. In addition, treatment of diabetic rats (group 3 and 4) with the aqueous extract of *H. hemerocallidea* did not show siginificant changes in the activity of SOD when compared to the diabetic control group 2 at *P<0.05*.
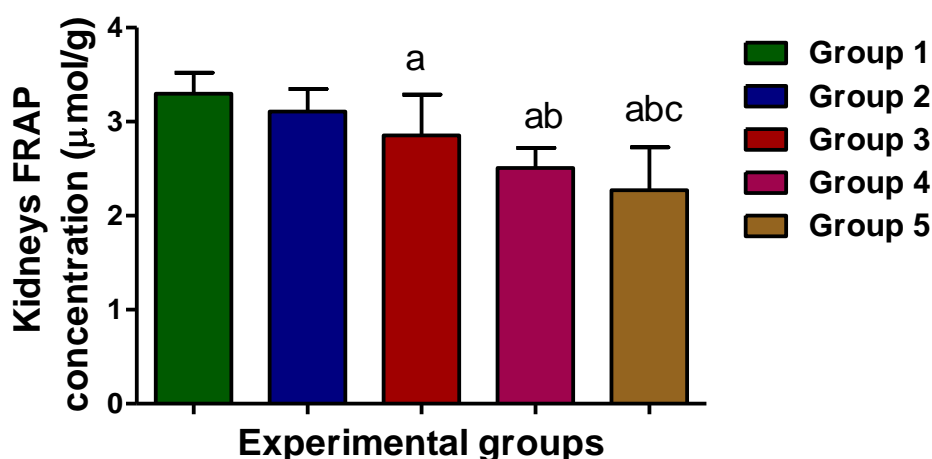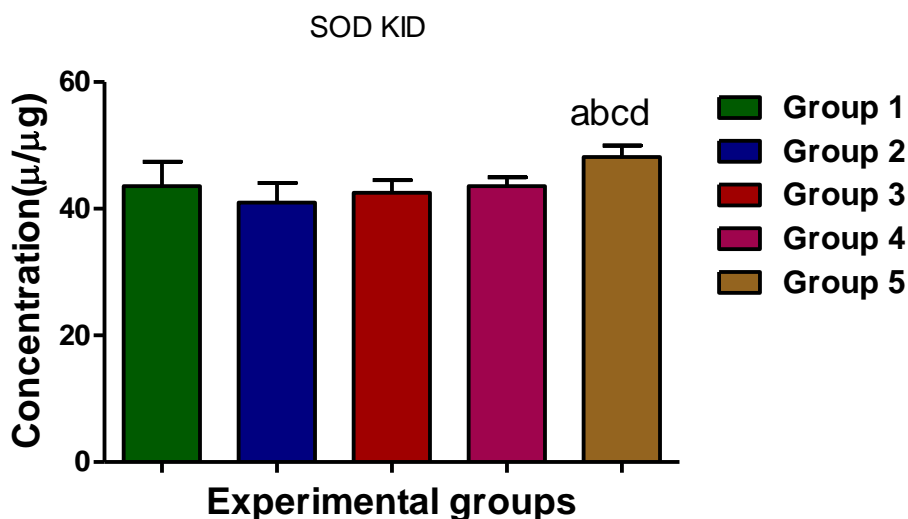

Figure 4.16: The effects of *H. hemerocallidea* on SOD activity in the kidneys

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents a significant difference of values in groups when compared to group 3 at *P <0.05*; (d) indicates significant difference of groups when compared to group 4 at *P <0.05*; U/ug: units per micro gram

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

4.9.4 Effects of *H. hemerocallidea* (aq) extract on the total GSH activity in the kidney tissue

Figure 4.17 indicates the effect of *H. hemerocallidea* extract on the activity of GSHt in the kidneys of STZ induced and normal rats. Administration of *H. hemerocallidea* extract in normal rats viz: group 5 produced a significant change in the activity of GSHt when compared to all groups at *P < 0.05*. There were no significant changes observed between groups 2, 3 and 4 when activities were compared to that of the normal control at *P < 0.05*.
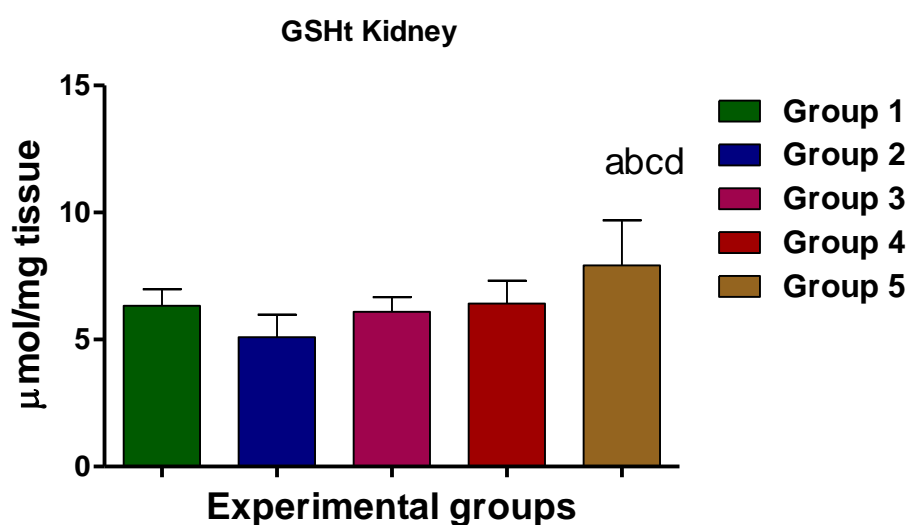
Figure 4.17: Effects of *H. hemerocallidea* (aq) extract on GSH activity in the kidney tissue

Abbreviations: (a) represents a significant difference when compared to group 1 control at *P <0.05*; (b) indicates significant difference of groups when compared to group 2 at *P <0.05* and (c) represents a significant difference of values in groups when compared to group 3 at *P <0.05*; (d) indicates significant difference of groups when compared to group 4 at *P <0.05*.

Experimental groups

Group 1   : Normal control;

Group 2   : Diabetic control;

Group 3   : Diabetic group fed with 800mg/kg of *H. hemerocallidea;*

Group 4   : Diabetic group fed with 200mg/kg of *H. hemerocallidea;*

Group 5   : Normal controls (non diabetic) fed with 800mg/kg of *H. hemerocallidea;*

Chapter 5
DISCUSSION

The rapid and intense shift from the native African lifestyles to those practised in the Western countries has greatly contributed to the rising epidemic trends of diabetes mellitus in Africa (Patel *et al.*, 2012). The cost of living and limited medical facilities in African regions still makes it very difficult if not impossible to effectively manage and treat diabetes, hence the need to study and identify alternative and complementary therapy such as phytotherapy which are cost effective, safe and could thus reduce mortality rate especially in rural areas of developing countries (Moniruzzaman *et al.*, 2012).

Phytomedicines possess potent therapeutic metabolites that have been linked to the medicinal values or medicinal activities of plants (Houcher *et al.*, 2007; Oguntibeju *et al.*, 2010; Oguntibeju *et al*, 2013; Ayepola *et al*, 2014a; Ayepola *et al.*, 2014b). These metabolites include but no limited to the following; carotenoids, flavanoids, alkaloids, polyphenols, terpernoids, sterols and glucosides (Patel *et al.*, 2012; Sudan *et al.*, 2014). These compounds work synergistically in hyperglycaemic states to exert hypoglycaemia. It has been reported that hypoglycaemia is achieved by these compounds' abilities to: reverse or reduce insulin resistance, increase the hepatic glucose output, decrease the rate of digestion, stimulate insulin output and inhibition of intestinal absorption (Aja *et al.*, 2015). *H. hemerocallidea* corm is rich in phytosterols (β- sitosterol) and hypoxide (that is converted to rooperol in the gut); these two compounds have been speculated to be responsible for its hypoglycaemic and antioxidant effects (Mahomed and Ojewole, 2003; Laporta *et al.*, 2007).

Proper monitoring and regulation of blood glucose levels in diabetes has been linked to delayed onset of microvascular and macrovascular complications (Cruz and Andre-Cetto, 2015). Induction of diabetes using STZ in rodents usually results in the destruction and necrosis of the beta-cells of the pancreas which consequently leads to diminished insulin release and elevated blood glucose levels (Eidi *et al.*, 2006). In the present study, STZ was the diabetogenic agent of choice because it enhances beta cell destruction by alkylating the DNA resulting in cellular necrosis of pancreatic cells in both rodents and humans (Ojewole, 2006; Irudayaraj *et al.*, 2012). In this study, significant elevation in the fasting blood glucose levels up to the diabetic range (Groups 2, 3 and 4) was observed 48 hours after STZ administration. Following treatment with the *H. hemerocallidea* (aq) extract, there was a significant decrease in fasting glucose levels in the treated groups 3 and 4. The greatest

percentage change of 73.3% in the fasting glucose levels was observed in the STZ group treated with 800mg/kg of the *H. hemerocallidea* (aq) extract followed by the group treated with a lower dose of 200mg/kg *H. hemerocallidea* (aq) extract with a 58.54% decrease. The glucose lowering effect observed in this study correlated with the results obtained by Ojewole (2006) in an acute study where he reported reduction in fasting glucose levels of diabetic rats using the same plant. Absence of glucose modification observed in group 5 (normal treated group) in this study is in contrast to proposition that consumption of *H. hemerocallidea* (aq) extract in healthy subjects does alter normal glucose metabolism to hypoglycemia. The results of the effect of the *H. hemerocallidea* (aq) extract in normal rats disagrees with the results obtained by Ojewole (2006) where he reported a decrease in fasted blood glucose levels in normal rats after treatment with the plant in an acute study. The mechanism of blood glucose level reduction is still obscure, however speculations exist that explains that the plant exerts a short lived antihyperglycaemic effect in which the mechanism mimics that of metformin (Musabayane *et al.*, 2005). Met is in the biguanide family and it acts by controlling hepatic glucose output achieved by reducing the rate of hepatic gluconeogenesis (Bailey, 2000).

Diabetes mellitus is associated with rapid weight loss as a consequence of uncontrolled catabolism of structural proteins as a compensatory response against the abnormal carbohydrate metabolism (Ravi *et al.*, 2004; Anupama *et al.*, 2012). Muscle atrophy in diabetic subjects is due to a combination of decreased protein synthesis, increased gluconeogenesis and increased protein degradation (Ono *et al.*, 2015). The results of the present study showed significant increase in the body weights of the normal untreated controls and group 5 (normal treated) rats because of the absence of pathology and undisturbed metabolism hence the continuous weight gain. Administration of both extracts of *H. hemerocallidea* (aq) in diabetic treated group did not significantly alter body weights despite the plant's glucose lowering effect (antihyperglycemic).

Diabetic nephropathy (DN) is labelled as a major complication of diabetes mellitus that affects about 40% of all diabetic patients (Gross *et al.*, 2005). A poor sustained glycemic control has been linked to the development of DN (Parchwani and Upadhyah, 2012). DN is characterised by both structural and functional features which include proteinuria, reduced glomerular filtration rate, renal hypertrophy, increased blood pressure and decreased creatinine clearance (Al Amin *et al.*, 2006; Eidi *et al.*, 2006). The emergence of renal dysfunction in this study was confirmed by increased kidney weights, an early event noted in the progression of glomerular pathology observed in the diabetic control (group 2) and the diabetic group treated with 800mg/kg *H. hemerocallidea* extract (group 3) as indicated in Fig

4.4. The kidney weights results from this study suggests that the increase in kidney weights in diabetic rats may have been caused by hyper filtration, aggregation of lymphocyte and fat infiltrations, glomerular hypertrophy which indicates increase in size and area of the glomeruli (Azemi *et al.*, 2012). It was observed that the high dose of *H. hemerocallidea* may have caused renal impairment since there were no significant differences in the kidney weights of group 3 rats when compared to group 2 diabetic control. Our findings in abnormal kidney function correlated with results of Musabayane *et al* (2005) where they reported that *H. hemerocallidea* reduced the glomerular filtration rate which implies the possible accumulation of toxic substances e.g. urea and also increases in serum creatinine levels. It appears that the plant induced a lower relative kidney weight (when compared to group 2 and 3) in the treated group 4 because of the lower dose and not in group 5 due to the absence of hyperglycemia induced effects. Elevated concentration of serum creatinine was seen in the diabetic control when compared to the normal control and group 5 indicating abnormal creatinine clearance by the kidneys and thus further confirming kidney dysfunction that can be also linked to decreased GFR as also reported by Musabayane *et al* (2005). The plant however, did not significantly alter serum creatinine levels in the treated groups when compared to the normal control.

The liver is the body's largest organ that is responsible for regulating and maintaining normal functioning of various physiological processes e.g. detoxification of molecules, protein deamination and carbohydrate metabolism. Subsequently, the liver has a fundamental role in the maintenance of normal blood glucose levels (Gometi *et al.*, 2014). The pathological effects of hyperglycemia and insulin resistance on the hepatic tissue have been demonstrated by elevated serum hepatic enzymes and liver hypertrophy (Haeri *et al.*, 2009). In this study, increased relative liver weights in the diabetic control and groups 3 and 4 may be due to hypoinsulinemia following STZ induction. Hypoinsulinemia triggers lypolysis which results in excess free fatty acids (FFAs) accumulations and storage in the hepatic tissue resulting in hypertrophy (Zafar and Nqvi, 2010; Gometi *et al.*, 2014). The *H. hemerocallidea* (aq) extract did not significantly ameliorate liver hypertrophy in the STZ treated groups. Amino transferases (AST, ALT) and ALP are hepatic enzymes that are assessed to monitor hepatic integrity and cardiac injuries (Eidi *et al.*, 2006). Elevation of serum amino transferases is linked to hepatic and cardiac pathologies which are both complications of diabetes. AST is mainly found in the cardiac, skeletal and hepatic tissues but found in minute quantities in red blood cells, thus excess levels in the serum strongly suggests injury of the liver tissues. On the other hand, ALT is found predominantly in the hepatocytes, therefore increased activities of both AST and ALT in the serum strongly point to hepatic injury (Eze *et al.*, 2012; Juarez- Rojop *et al.*, 2012).

In previous literature, increased serum activity of ALP in hyperglycaemic environment was linked to the peroxidation of lipids in the cell membranes of hepatic cells (Uboh *et al.*, 2010) whereas leakage of AST and ALT from the hepatocytes' cytosol into the blood was reported to be caused by the disruption of hepatocytes' cell membranes as an effect of the accumulations of toxic free fatty acids (FFAs) (products of lypolysis due to hypoinsulinemia) (Gometi *et al.*, 2014). In this current study, there was a marked increase in serum activities of hepatic enzymes in group 2 (increase in all hepatic enzymes), group 3 (increase in AST and ALP) and group 4 (increase in ALP) when compared to the normal control group. The increase in serum hepatic enzymes in the STZ induced groups (2, 3 and 4) can not only be explained by the hyperglycemia mediated hepatic injury but also by the hepatotoxic effects of STZ itself since the liver is the organ responsible for drug metabolism (Zafar and Naqvi, 2010). This implies that abnormal liver function results are as a result of the combined hepatotoxic effects of STZ and excess glucose levels. It is logical to say that both the doses of 800mg/kg and 200mg/kg of the *H. hemerocallidea* (aq) extract could not prevent leakage of hepatic enzymes (AST and ALP) and ALP respectively from the hepatocytes as there were significant higher activities when compared to the normal control group. The hepatoprotective and ameliorative effects of the plant were mostly observed in group 4, which received 200mg/kg of the extract as there was only ALP leakage.

Albumin is a major protein in serum that is produced by the liver; abnormal levels of albumin in serum may suggest hepatic damage. It plays a fundamental role in the maintenance of blood osmolality and transportation of molecules (O'connell *et al.*, 2005). Serum levels of albumin can be reduced as a result of renal loss, malnutrition, pregnancy and trauma; therefore it is necessary to rule out these factors before a diagnosis can be made from reduced albumin levels. In most cases increased serum levels of albumin occur as a consequence of severe dehydration (O'connell *et al.*, 2005). Decreased levels of both albumin and total protein in diabetic patients may occur as a result of decreased protein synthesis rate. Protein synthesis in diabetics is derailled because the mechanisms involved in protein synthesis require ATP from glucose metabolism, in which glucose metabolism is attenuated in diabetes. Gluconeogenesis or protein catabolism occurs in diabetes in attempts to balance ATP production (Murugan and Pari, 2007). In the present study, there were significant decreases in the serum levels of both total protein and albumin in groups 2, 3 and 4 when compared to the normal control and group 5. Reduced levels of the proteins may be due to hyperglycemia which targets proteins that would then be utilised in advanced glycation end products (AGEs) formation (Anupama *et al.*, 2012). Other reasons to explain the decrease involves; the increase in protein catabolism, decreased synthesis of proteins,

liver damage and renal loss (polyuria) due to renal impairment (in this study renal dysfunction was confirmed by kidney hypertrophy). It was observed that both doses of the plant did not redeem albumin and total protein levels in diabetic treated rats. The results differ from that obtained by Ayepola *et al* (2014) where the authors observed normalised albumin and total protein levels in *Kolaviron* treated diabetic rats. Decreased levels of albumin and total protein were also confirmed by weight loss in the diabetic controls and failure to gain weight in group 3 and group 4 rats, all together pointing to abnormal carbohydrate metabolism.

Oxidative stress has been documented as a critical participant in the pathogenesis of various diseases as it causes damage to cellular components and therefore loss of cellular function (Pitocco *et al*., 2010) Significant effects of oxidative stress are seen in the development of various pathologies such as cardio-vascular diseases, diabetes, cancer and some neurological diseases (Ghasemzadeh *et al*., 2010; Ayepola *et al*., 2014). Biological systems possess a complex of defence mechanisms that work together in combating the detrimental effects of reactive oxygen species (Azemi *et al*., 2012). The complex defence system consists of enzymes and proteins such as catalase, SOD, glutathione peroxidase, glutathione, albumin and uric acid. During oxidative stress conditions, the sources of endogenous antioxidants are restricted hence in-corporation of exogenous antioxidants is essential to replenish the total antioxidant pool. Exogenous antioxidants derived from dietary therapy and plant materials include vitamin C, vitamin E, flavanoids, flavanols, polyphenols, carotenoids, steroids, tepernoids, glucosides and sterols (Birden, 2012). Dietary and phyto constituents have been associated with delayed development of diabetic complications and prolonged longevity.

In diabetes mellitus, the reactive oxygen species scavenging power of antioxidants becomes weakened resulting in diabetes induced oxidative stress (Houcher *et al*., 2007). In this study, the total antioxidant capacity in both liver and kidney tissues was assessed by the ORAC method. The ORAC is an oxidation inhibition based method that measures the ability of a molecule or specimen to inhibit the oxidation of a fluorescent probe derived from peroxyl radicals; it also measures the hydrophilic chain breaking antioxidant capacity. If present, the antioxidants bind to the radical and the association results in a delayed fluorescent decay. In the present study, there was a significant increase in liver oxygen radical absorbance capacity in diabetic group 3 (treated with 800mg/kg) when compared to the diabetic control, group 2. The decrease in ORAC levels confirms poor glycemic control and ROS accumulation in the diabetic control group (Mancino *et al*., 2011). There was no significant difference in ORAC levels of group 3 and group 5 when both were compared to the normal control group 1, which suggests the antioxidant power of the *H. hemerocallidea* at a dose of

800mg/kg. There were no statistical significant differences in the absorbance capacities between the diabetic control group 2 and the rats in group 4 (200 mg/kg) which indicated a lower antioxidant activity at 200 mg/kg dosage. However in the kidney tissue, there were no significant differences in ORAC levels between group 3 (diabetic group treated with 800 mg/kg of the *H. hemerocallidea*) and the diabetic control group 2. In both groups the ORAC levels were decreased when compared to group 1, group 4 and group 5. It can be suggested that in diabetic kidneys, the dosage of 800mg/kg of the *H. hemerecollidea* ca not ameliorate the oxidative stress because of its potential toxic effects; the results are in line with the findings of Musabayane (2014) who further reported detrimental effects of *H. hemerecollidea* in the kidneys. More over, the antioxidant capacity in the diabetic group treated with 200 mg/kg was increased with no significant differences to group 1 and group 5. Therefore, it is possible to suggest that the 200 mg/kg dosage of *H. hemerecollidea* induces desirable antioxidant effects than the 800 mg/kg dosage in the kidneys.

The ferric reducing antioxidant power assay (FRAP) was also performed to assess and confirm the presence of functional recovery in the treated groups after induction of diabetes with STZ. There was increased ferric reducing antioxidant power in the liver of the treated groups 3, 4 and 5 when compared to untreated group 1 normal control and the diabetic control (group 2). It appears that at both dosages of the plant in groups 3 and 4, the reducing antioxidant power of the plant was rejuvenated when compared to the diabetic control group. There was no significant difference in FRAP values between group 1 and group 2. The seemingly decreased levels of FRAP in the normal control group (group1) could be due to the non-pathological state hence no extra demand for antioxidants to curtail less amounts of ROS. Nevertheless, in the kidneys, there were no significant differences seen in group 2 (diabetic control) when compared to group 1 (normal control). The unexpected increase in FRAP in the diabetic control could be due to chronic hyperglycemia that occurred after STZ administration. Increase in FRAP in diabetic rats was linked to ketosis by Sasvari and Nyakas (2003). In their study, the authors reported that oxidative metabolism is dependant on time period. In addition, they also reported that during acute hyperglycemia, the FRAP levels tend to decrease while it later increases during chronic hyperglycemic phases which could be the case in this current study. Increase in enzyme activities indicates increase in demand to meet up with oxidative stress requirement (Houcher *et al.*, 2007). There was no significant difference in the FRAP levels of group 3 when compared to group 2 which indicates combined effects of hyperglycemia and toxicity of the plant which negatively influenced the FRAP activity in the kidney tissue.

Catalase (CAT) is an endogenous antioxidant that highly determines hepatic antioxidant status. It catalyses the conversion of H_2O_2 to water and oxygen and renders inactive the harmful peroxidases (Venkateswaran and Pari, 2003). The hepatic catalase results of this study showed a significant decrease in the catalase activity of group 2 diabetic rats when compared to all the groups. The results of this study correlated with the study of Ayeleso *et al* (2014) in which they reported a significant decrease in hepatic catalase activity in diabetic rats when compared to the groups treated with red palm oil. The decrease in catalase activity in the diabetic hepatic tissue signifies the failure of catalase to flush out hydrogen peroxide resulting in increased oxidative damage. No clear changes were noted in diabetic treated groups 3 and 4 when compared to the normal control and in group 5, thus indicating the ameliorative and upregulatory effects of the plant on catalase activity. In diabetic treated groups, both extracts of *H. hemerocallidea* increased the antioxidant activity of catalase suggesting the plant's potent antioxidant activities as previously reported by Larporta *et al* (2007). On the contrary, the catalase activity in the diabetic kidney tissue (Group 2) was increased when activity was compared to group 4 and group 5 whereas there was no significant difference when the activity was compared to the diabetic treated group 3 (800 mg/kg) and the normal control group. The increase in catalase activity in the diabetic group was in line with the results that were reported by Qujeq and Rezvani (2007); they suggested that the increase in catalase activity in diabetic rats was due to the uncontrolled ROS production as a consequence of hyperglycaemia which upregulated the expression of catalase. In addition, increase in catalase gene expression in diabetic rats has been reported to be a natural response of the cell during oxidative stress (Limaye *et al.*, 2003; Yilmaz *et al.*, 2004). Absence of a significant difference of catalase results in Group 2 diabetic control and group 3 rats could be possibly due to compensatory responses as a result of severe hyperglycemia. It can be suggested that the plant may not have kidney catalase stimulatory effects since at the same dose of 800mg/kg in normal treated rats; there was no elevation of catalase activity.

Superoxide dismutases are the first line of defense antioxidant enzymes that target the superoxide anion (Venkateswaran and Pari, 2003). SOD enzyme catalyses the dismutation of the superoxide anion to hydrogen peroxide and oxygen. Superoxide dismutases are mainly found in the cytosol attached to metal ions such as copper, zinc and manganese. The products of superoxide dismutation are further catalysed by catalase into water and oxygen (Heidarian and Soofoniya, 2011). It has been reported that hyperglycemia causes significant decreases in SOD activities in the tissues of diabetic rats as a result of inactivation of SOD by the hydrogen peroxide or by glycation (Kumawat *et al.*, 2010). In disagreement to Kumawat *et al* (2010), there was no significant difference in the SOD activities in the liver

tissue of all groups when compared to the normal control group 1. While this study showed non-significant changes in the SOD activitiy in the diabetic group when compared to the normal control, other studies measured either reduction or increase in its activities. Dias *et al* (2005) however, reported that the discrepancies encountered when assessing antioxidant enzyme activities were due to differences in tissue specificity, variation in disease severity and duration of the disease. Both doses of the plant did not have substantial effects on the SOD activities in the diabetic treated groups. In the kidney tissue, the normal treated group 5 showed significant increased SOD activitiy when compared to the activities of all groups, which suggest the ability of *H. hemerocallidea* to boost renal SOD activities thereby improving the antioxidative status when compared to the normal untreated group 1. The activities of SOD in groups 2, 3 and 4 were found to be insignificant when compared to the normal control (group 1).

GSH is another important endogenous antioxidant and a cofactor of some enzymes taking part in the reduction of ROS hence it has been regarded as a marker of free radical damage. The reduction of GSH levels in diabetic animals was linked to abnormally high levels of glucose that finally induces oxidative stress (Can *et al.*, 2004). Therefore, in diabetic conditions; overusage and reduction of GSH concentration indicate the overpowering effects of ROS over antioxidants (Hamdy, 2012). The hepatic GSHt activity in diabetic controls was significantly lower when compared to group 1 and group 5. Decrease in the hepatic GSHt level was linked to the depletion of GSHt stores possibly encountered during free radical scavenging in response to hyperglycemia induced oxidative stress. Treatment of diabetic induced group 3 and group 4 rats with *H. hemerocallidea* (aq) extract showed increase in the GSHt activity though the increase was not significant when compared to the diabetic control group. The *H. hemerocallidea* (aq) extract did not significantly alter GSHt levels in the treated groups. The significant elevation in GSHt activity was observed in the kidney tissue of the normal treated group (group 5) when compared to all the groups. This study did not show any appreciable changes in the GSHt levels of the diabetic treated groups when compared to the non-treated diabetic control.

Chapter 6

CONCLUSION AND RECOMMENDATIONS

The aim of this study was to examine the modulatory effects of *H. hemerocallidea* aqueous extract in hyperglycaemic induced Wistar rats focusing on hepatic and kidney tissues. It was observed that the extract significantly reduced fasting blood glucose levels in diabetic treated groups and that the reduction in blood glucose level was dose-dependent. We also investigated the effects of *H. hemerocallidea* (aq) on oxidative stress biomarkers and biochemical parameters in both diabetic and non-diabetic rats.

The results of this study showed that *H. hemerocallidea* corms could have antioxidant stimulatory effects in the hepatic tissue. In the hepatic tissue of rats treated with the plant extract, an overall increase in antioxidant activities (ORAC, FRAP, catalase and GSHt) was observed with the greatest antioxidant effect seen in the group treated with 800mg/kg. It was noted that although the 800mg/kg extract imposed a higher antioxidant activity in the hepatic tissue and despite the excellent glucose lowering effect at this dose. An inauspicious effect at this higher dose in the kidney tissue was observed in Group 3. Both dosages of *H. hemerocallidea* (aq) did not show desirable alteration on biochemical parameters (albumin, total protein and hepatic enzymes) when compared to the normal control.

Based on the findings of this study, it can be presumed that the mechanism of action of the plant extract involved enhanced antioxidant activity and antihyperglycemic effect in the group that experienced greater glucose reduction.

Further studies on this plant should therefore aim to investigate the exact mechanism by which antihyperglycaemia is effected in diabetic state. In-depth experimental studies of the effects of *H. hemerocallidea* on other organs such as the brain, cardiac, pancreas could possibly provide further understanding on its effects especially in a diabetic model. In addition to *in vivo* studies, *in vitro* studies in different hyperglycemic induced human cell lines on the effect of this plant could give a better understanding of the therapeutic activities and efficacy of the plant since *in vivo* studies alone can not provide conclusive results.

Although it is evident that *H. hemerocallidea* corms confer beneficial health effects which include antihyperglycemia and hepatic antioxidant effects, its negative effects in the kidneys observed in this study and also as reported by Musabayane (2005) and (2014) should not be ignored. Hence, future pharmacological investigations are needed to identify and isolate safe and active components that could be responsible for the antihyperglycemic and antioxidant properties of *H. hemerocallidea*.

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