

**MODULATORY EFFECTS OF *MORINGA OLEIFERA* EXTRACTS ON
STREPTOZOTOCIN-INDUCED DIABETES IN MALE WISTAR RATS.**

by

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Declaration

I, Elizabeth lfe Omodanisi, declare that the contents of this thesis represent my own unaided work and that the thesis has not 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.

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Abstract

Diabetes mellitus (DM) is characterized by deficiency in insulin resulting in hyperglycaemia with metabolic alterations in carbohydrate, lipid and protein. DM has been associated with increased formation of reactive oxygen species (ROS) and inflammatory mediators. Many drugs have been designed for its treatment and management; however, limitations persist in the use of anti-hyperglycemic medications due to numerous side effects, high cost, limited action and secondary failure rates. *Moringa oleifera* (MO) tree is distributed in the tropics and subtropics and has been found to be very nutritious with a variety of applications. This plant has been reported to possess antidiabetic, antioxidant and other medicinal properties which may be helpful in managing diabetes and its associated complications.

This study investigated the antioxidant status, antidiabetic, antilipidemic, anti-inflammatory, anti-apoptotic properties and phytochemical constituents of the leaf extract of MO (250 mg/kg). Diabetes was induced in Wistar rats by a single intraperitoneal injection of streptozotocin (STZ) in buffered citrate (0.1, pH 4.5). Forty-eight Wistar rats were randomly divided into four (4) groups and treated for six weeks: group one- non-diabetic control (Control), group two- non-diabetic *Moringa* treated (*Moringa*), group three- diabetic control (Diabetic) and group four- diabetic *Moringa* treated (Diabetic + *Moringa*).

Methanol, aqueous and petroleum ether extract of MO leaves were evaluated for its antioxidant and phytochemical contents. Assays for total antioxidant capacity such as trolox equivalence antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), flavonoids, flavonols and total polyphenols content were analysed. Other parameters analysed include glucose level; glycated haemoglobin level; hepatic biomarkers; endogenous antioxidants (SOD, CAT, GSH, GPx) in the liver; kidney and erythrocytes; inflammatory biomarkers in the serum, liver and kidney; high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol (TC) in serum. Assessment of apoptotic cell death biomarkers (caspase 3, caspase 9, BCL-2, NFK β , p53) in the liver and kidney were performed. Histopathological analysis was conducted on the liver, kidney and pancreatic sections.

In vitro results showed that aqueous and methanol extract of MO demonstrated a high antioxidant capacity, phenolic contents and revealed more chemical constituents than the petroleum ether extract. HPLC analysis of the leaf extract indicated the presence of flavonoids: quercetin, rutin and myricetin and phenolic acids. High levels of polyphenols, flavonols and alkaloids were reported in MO extracts.

Treatment with MO in normal and diabetic rats daily for six weeks resulted in significant ($p < 0.05$) decrease in glucose and glycated haemoglobin levels. Liver and kidney size which

increased in diabetic rats, decreased significantly ($p < 0.05$) after treatment with MO. Pancreas size showed significant ($p < 0.05$) decrease in diabetic rats and increased significantly ($p < 0.05$) after MO administration. Similarly, serum albumin level increased in non-diabetic and diabetic groups after MO treatment. Also, a significantly increased level of T-bilirubin in diabetic groups relative to normal control rats which reduced greatly after MO administration was observed.

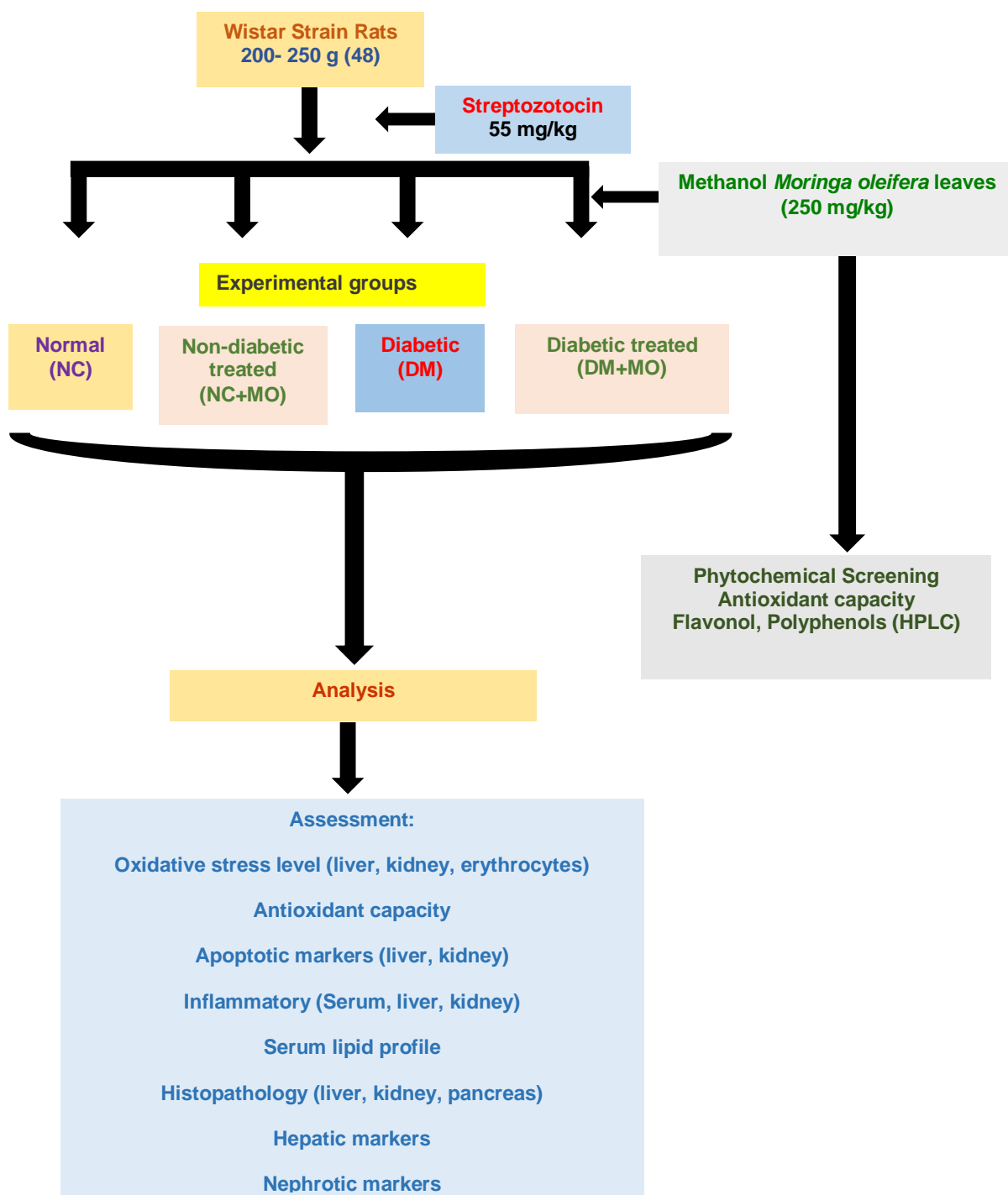
Serum lipid profile: LDL and TC levels were increased in rats exposed to STZ. HDL level decreased in diabetic rats when compared to normal control. The activities of MO extracts was shown to lower TC and LDL levels. HDL level also increased after MO administration. Similarly, lipid peroxidation (MDA) level significantly ($p < 0.05$) decreased in the diabetic group following MO treatment. An observable improvement was seen in the antioxidant enzyme system. Activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and concentration of glutathione (GSH) were restored or increased in the homogenate of the liver, kidney, and erythrocytes, indicative of the protective effect of MO in diabetic and non-diabetic rats.

The expression of cell death markers (caspase 3, caspase 9, BCL-2, NFK β , p53) showed remarkable improvement after treatment with MO relative to the non-diabetic control. A significant ($p < 0.05$) reduction in inflammatory cytokines (IL-1 α , IL-6, IL-12, IL-18, TNF- α) and (chemokine MCP-1 concentrations) were observed in the serum, liver, and kidney of non-diabetic and diabetic treated groups. Histopathological sections of the liver, kidney and pancreas of diabetic rats revealed severe damage which showed significant improvements after MO treatment. Liver, kidney and pancreatic histological sections revealed the protective effect of MO in both non-diabetic and diabetic rats.

MO exerted modulatory effects in STZ-induced diabetes by its antidiabetic, hypoglycemic, antioxidant, anti-inflammatory, anti-apoptotic and anti-lipidemic activities and offered protective effects against diabetic-induced nephrotoxicity and hepatotoxicity, but equally improved antioxidant status. The study concluded that MO could play a significant role in the early treatment and management of diabetes that pharmaceutical industry should consider it in the future as a possible therapeutic agent.

Keywords - MO, diabetes, glucose, methanol extract, streptozotocin, antioxidant, inflammation, hyperglycemia, nephrotoxicity, hepatotoxicity, antilipidemic, phytochemical, anti-apoptotic, liver, kidney, serum, pancreas.

Graphical abstract



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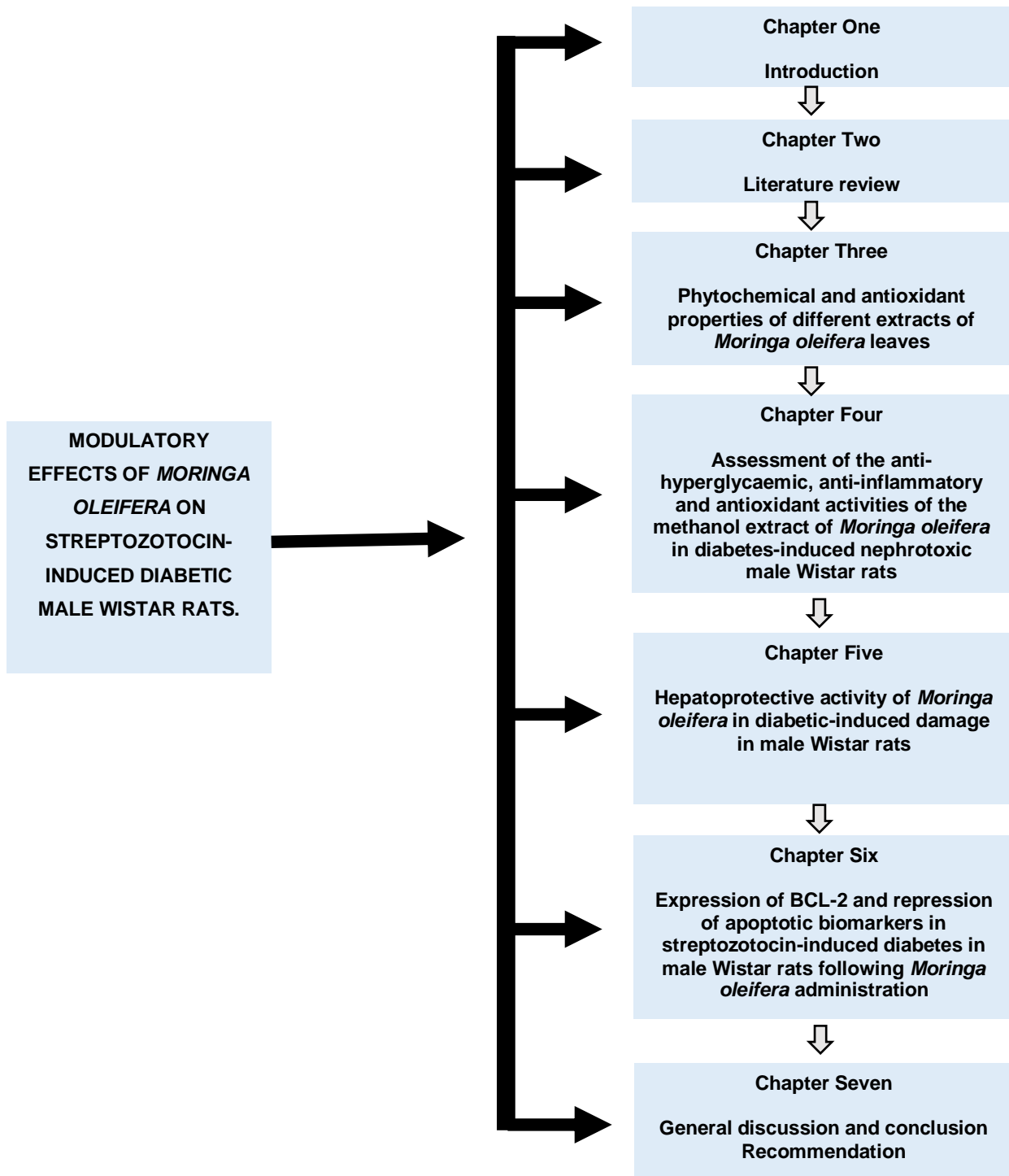
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Dedication

This project is dedicated to God for his sustenance and blessings throughout my research and my beloved parents Rev. (Dr) Moses and Rev Mrs. Grace Omodanisi for your sponsorship, sacrifices and encouragements. I love you.

Biographical sketch



Preface

This thesis consists of seven chapters, written in article style format according to the style of the journal where it has been submitted for review. The research results are presented and discussed in chapters three, four, five and six. The general discussion, conclusion, and recommendation are summarized in chapter seven.

Chapter one is the introductory chapter. It gives a brief introduction on the meaning of diabetes and the worldwide endemic nature of the disease.

Chapter two presents the literature review which provides an overview of diabetes, oxidative stress and other complications. It also highlights the modulatory and beneficial effect of *Moringa oleifera*. A review paper which forms part of the literature review has been accepted for publication in **Tropical Journal of Pharmaceutical Research**.

Chapter three presents the experimental results of the study titled “Phytochemical and antioxidant properties of different extracts of *Moringa oleifera* leaves” has been submitted for publication to **South African Journal of Botany**.

Chapter four presents the experimental work titled “Assessment of the anti-hyperglycaemic, anti-inflammatory and antioxidant activities of the methanol extract of *Moringa oleifera* in diabetes-induced nephrotoxic male Wistar rats” has been published by **Molecules**.

Chapter five presents the experimental work titled “Hepatoprotective activity of *Moringa oleifera* in diabetic-induced damage in male Wistar rats” has been published by **Pharmacognosy Research**.

Chapter six which focuses on the experimental work of the research titled “Expression of BCL-2 and repression of apoptotic biomarkers in streptozotocin-induced diabetes in male Wistar rats following *Moringa oleifera* administration” has been submitted for publication to **Journal of Complementary and Integrative Medicine**.

Chapter seven: The general discussion and conclusion of all the experiment are summarized in the chapter. The chapter also provides relevant recommendations for future research.

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Glossary

Abbreviations Explanation

AAPH -	Azobis (2-amidino-propane) dihydrochloride
ABTS -	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ADP -	Adenosine diphosphate
AGEs -	Advanced glycated end products
ALT -	Alanine aminotransferase
ANOVA -	Analysis of variance
AQU-	Aqueous
AST -	Aspartate aminotransferase
ATP -	Adenosine triphosphate
AUC-	Area under curve
BCL-2-	B-cell lymphoma 2
CAT -	Catalase
CCl ₄ -	Carbon tetrachloride
c-JNK	c-Jun N-terminal kinase
CVD -	Cardiovascular diseases
DPPH-	2,2-diphenyl-1-picrylhydrazyl
DM -	Diabetes mellitus
DMACA -	p-Dimethylaminocinnamaldehyde
DM + MO-	<i>Moringa oleifera</i> -treated diabetic rats
DNA -	Deoxy ribonucleic acid
EDTA -	Ethylenediaminetetraacetic acid
EGF-	Endothelia growth factor
FeCl ₃ -	Iron (III) chloride
FRAP-	Ferric reducing antioxidant power
GGT-	Gamma-glutamyl transferase
GPx-	Glutathione peroxidase

GR-	Glutathione reductase
GSH-	Reduced glutathione
HOCl-	Hydrogen oxychloride
H ₂ O ₂ -	Hydrogen peroxide
H ₂ SO ₄ -	Tetraoxosulphate (VI) acid
HbA1c-	Glycosylated haemoglobin
HDL-	High density lipoprotein
HNO ₂ -	Nitrous oxide
HOCl-	Hydrochlorous acid
HRO ₂ -	Hydroperoxyl
IDDM-	Insulin-dependent diabetes mellitus
IHC	Immunohistochemistry
IL-1 α-	Interleukin-1 alpha
IL-6-	Interleukin-6
IL-12-	Interleukin-12
IL-18-	Interleukin-18
LDH-	Lactate dehydrogenase
LDL-	Low density lipoprotein xviii
LPO-	Lipid peroxidation
MAPK-	Mitogen-activated protein kinase
MCP-1-	Monocyte chemotactic protein-1
MDA-	Malondialdehyde
MO-	<i>Moringa oleifera</i>
MET-	Methanol
NAD(H)-	Nicotinamide adenine dinucleotide
NADP(H)-	Nicotinamide adenine dinucleotide phosphate
NFκ-B	Nuclear factor kappa-light-chain-enhancer of activated β- cells
NIDDM -	Non-insulin-dependent diabetes mellitus
NO-	Nitric oxide

NC-	Normal control
NO ₂ -	Nitrogen dioxide
O ₂ -	Superoxide anion
OH-	Hydroxyl anion
ONOO-	Peroxynitrite
ORAC-	Oxygen radical absorbance capacity
PET-	Petroleum ether
PCA-	Perchloric acid
PK-	Pyruvate kinase
PKC -	Protein kinase C
PUFA -	Polyunsaturated fatty acids
RNS-	Reactive nitrogen species
RO ₂ -	Peroxyl
RONOO-	Alkyl peroxy nitrates
ROS -	Reactive oxygen species
SD -	Standard deviation
SOD-	Superoxide dismutase
SRC-	Standard rat chow
STZ -	Streptozotocin
TBA -	Thiobarbituric acid
TBARS-	Thiobarbituric acid reactive substances
TC-	Total cholesterol
TEAC-	Trolox equivalence antioxidant capacity
TG-	Triglycerides
TNF α -	Tumor necrosis factor alpha
TPTZ-	Tripyridyl triazine
WHO-	World Health Organization

Definition of terms

Antioxidants	are chemicals derived from plant, vitamins and other nutrients which guard the cells of the body system from damaging effects caused by free radicals, thus acting as a free radical scavenger. It also inhibits the oxidation of other molecules.
Apoptosis	is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area.
Biomarker	is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention.
Free radicals	are any chemical species capable of independent existence with an unpaired number of electrons and can be formed when oxygen interacts with certain molecules. They are highly reactive species, which comprises of reactive nitrogen species (RNS) and reactive oxygen species (ROS).
Reactive Oxygen species	Reactive Oxygen Species (ROS) encompass a variety of partially reduced metabolites of oxygen which can be classified into oxygen-derived free radicals and oxygen-centred non-radicals.
Immunohistochemistry	is an important application of monoclonal as well as polyclonal antibodies to determine the tissue distribution of an antigen of interest in health and disease.
Inflammation	is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants, and is a protective response involving immune cells, blood vessels, and molecular mediators.
Modulatory	means to regulate by or adjust to a certain measure or proportion.
Oxidative stress	is an imbalance between the generation of reactive oxygen species and the activity of the antioxidant defences in favour of the oxidant. Oxidative stress can lead to cell damage and cell death.

CHAPTER ONE

INTRODUCTION

1 Background of study

To be healthy is to be in a state of complete physical, mental, and social well-being; it is being free from any signs of disease (WHO, 2013). Diabetes mellitus (DM) is a major public health problem challenging the well-being of individuals globally (Harries et al., 2016). The International Diabetic Federation (IDF), reported that over 246 million people globally suffer from diabetes which is expected to rise to 380 million by the year 2026 (IDF, 2006). DM is a metabolic disorder of multiple aetiologies, and it is characterized by persistent high blood sugar (hyperglycemia) resulting from defects in insulin production, insulin actions or both (American Diabetes Association, 2012). Hyperglycemia in DM has been associated with increased formation of ROS which are chemically reactive molecules containing oxygen that damage important tissues and organs in the body with the upregulation of inflammatory mediators (Van den Oever et al., 2010; Anzar, 2013).

Consequently, if hyperglycemia is left untreated, it can lead to severe complications. There is an incipient worldwide epidemic of diabetes that can be linked to a rapid increase in weight, obesity and sedentary lifestyle (Prentice & Moore, 2005). The general prognosis is that there will be continual increase in the global prevalence of diabetes, possibly turning to be the seventh prominent cause of death worldwide by the year 2030, with total death from diabetes projected to rise by more than 50% in the next 10 years (Ansari & Dash, 2013; Lopez & Mathers, 2013). Many drugs have been formulated for the treatment of DM. However, there are limitations in the use of anti-hyperglycemic medications resulting from the side effects, high cost, limited action and secondary failure rates (Baggio & Drucker, 2007).

There are various reports which indicate increase in reactive oxygen species (ROS) generation and systemic markers of inflammation in both types of diabetes showing a potential relationship between DM and inflammation and ROS (Luqman et al., 2012). Increase in

inflammatory cytokines such as IL-1, IL-6, IL-18 and TNF- α has been observed in the blood of patients with diabetes (Esposito et al., 2002; Alexandraki et al., 2008). Chronic hyperglycemia and increased oxidative stress play a vital role in the development of secondary diabetic complications such as renal and hepatic injury (Huang et al., 2002; Harrison 2006; Kim et al., 2009).

The use of medicinal plants could be an alternative means to improve health care globally particularly in poor resource countries (Malviya et al., 2010). Medicinal plants are locally available, easily accessible regardless of social status (Maghrani et al., 2005). Phytochemicals (plant chemicals with protective or disease preventive activity) which emanate from plants offer a notable prospect for the exploration of new varieties of therapeutics. As a result, efforts are being geared globally towards the exploitation of these medicinal plants which possess significant amount of phytochemicals exhibiting diverse beneficial effects in tackling diabetes and associated complications (Atmakuri & Dathi, 2010).

1.1 Statement of research problem

The increasing prevalence of diabetes and its complications in rapid successions has become a major concern worldwide and there is a need to prevent or manage the disease (Stumvoll et al., 2005). Various hypoglycemic medications have been formulated, but have been found to display serious side-effects, prompting the need for the development of indigenous and inexpensive herbal sources for treatment and management of diabetes (Atmakuri & Dathi, 2010). These limitations have stimulated the need to explore the potential anti-diabetic, antioxidant and anti-inflammatory activities of *Moringa oleifera* (MO). The leaf of this plant has been identified to possess an abundance of antioxidant and phytochemicals with medicinal activities and could possibly pose as effective constituents of therapeutic agents for the treatment and management of diabetes. Hence, this research was designed to examine the modulatory effects of MO for the treatment and management of diabetes.

MO has been found to show hypolipidaemic, antimicrobial, antifungal, antituberculosis, anthelmintic and analgesic properties (Iqbal and Bhangar, 2006; Chumark et al., 2008; Trapti et al., 2009). However, these studies are limited in scope and in some cases their results are inconclusive. Also, due to its numerous health benefits, it is a strong reason to investigate the potential treatment regimen of the plant on diabetes using advanced biochemical techniques. This study elucidated the activities of MO extract in a diabetic animal model. It equally provided a new insight into the understanding of the mechanisms of action of MO extract and in the treatment of diabetes. No doubt, it throws more light in establishing the safest modalities in its administration providing stunning capabilities that could potentially revolutionise pharmacological products in the treatment and management of diabetes.

1.2 Research questions

1. What are the concentrations of polyphenols, alkaloids, flavonoids and flavonols content and other phytochemicals present in MO plant extract?
2. What are the potential effects of MO on serum lipid profile and hepatic parameters in diabetic and non-diabetic male Wistar rats?
3. Does MO have potential effects on the oxidative status and antioxidant capacity of diabetic and non-diabetic male Wistar rats?
4. What are the potential effects of MO on inflammatory biomarkers in the serum, liver and kidney of diabetic and non-diabetic male Wistar rats?
5. Would MO demonstrate any potential effects on expression and activity of apoptotic proteins in diabetic rats and non-diabetic male Wistar rats?
6. What possible negative effects could it have on cells in various organs such as the pancreas, liver and kidney?

1.3 Hypothesis

MO in STZ-induced diabetes exerts its anti-diabetic, hypoglycemic, antioxidant, anti-inflammatory, anti-apoptotic and antilipidemic properties thereby reducing diabetic complications.

1.4 Aim and objectives of study

1.4.1 Aim

The research investigated the protective effects of MO leaves extract in streptozotocin (STZ)-induced diabetes on rat liver, kidney, pancreas and blood. The study further examined specific diabetic, oxidative stress and apoptotic, inflammatory and pathological markers in these tissues. The overall aim, therefore, is to explore the potential benefits of MO which can be used in the prevention and management of diabetes and as a component for the pharmaceutical industry in treating diabetes in the near future especially in developing countries.

1.4.2 Objectives

1. To determine the phytochemical properties of MO leaves.
2. To monitor lipid profile such as high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and total cholesterol (TC).
3. To determine liver and kidney function test together with enzymatic and non-enzymatic antioxidant status.
4. To investigate the histological section of the liver, kidney, and pancreas of both diabetic and non-diabetic rats.
5. To assess apoptotic cell death biomarkers and inflammatory biomarkers.

1.5 Ethical consideration

This study received approval from the Faculty of Health and Wellness Sciences, Research Ethics Committee (REC) of Cape Peninsula University of Technology, South Africa (CPUT/HW-REC 2014/AO8).

1.6 Animal care

Forty-eight adult male Wistar rats between 200-250 were randomly assigned into two experimental groups and two control groups after rats were confirmed diabetic fasting blood glucose of >18 mmol/L. Rats were purchased from Charles River (Margate, UK), housed and treated at the animal facility of Stellenbosch University, Cape Town, South Africa. All animals received humane care in accordance to the principles of National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) of the National Academy of Sciences.

1.7 Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) to determine the significant difference between experimental group means, followed by post hoc comparisons test. This analysis was performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, La Jolla, CA, USA). Values were represented as mean \pm standard deviation and criterion for significance was set at p -value < 0.05.

CHAPTER TWO

LITERATURE REVIEW

2 INTRODUCTION

2.1 Diabetes

The word diabetes originates from a Greek word "siphon". Aretus the Cappadocian described the condition known as *diabainein* - passing too much water (polyuria). Thomas Willis added mellitus to the term in 1675, drawing reference from the term "Mel" which means "honey" in Latin. Taking cognizance of the fact that the blood and urine of diabetic individuals have excess glucose, DM could literally be taken to mean "siphoning off sweet water". The term "Sweet Urine Disease" was coined when ants were observed to be attracted to some individuals' urine, because of glucose content in the urine (Mandal, 2012). Diabetes is associated with the destruction of pancreatic β -cells which consequently leads to insulin deficiency resulting in insulin resistance (American Diabetes Association, 2010).

Diabetes is characterised by chronic hyperglycemia, a very common metabolic abnormality among diabetic individuals inducing increased ROS generation which subsequently and oxidative stress (Evans et al., 2002). Hyperglycemia is associated with failure of various organs; the liver, eyes, kidneys, blood vessels, nerves, heart, and damage to macromolecules (Dutta et al., 2008). Hyperglycemia causes oxidative stress via several mechanisms leading to a rise in the generation of advanced glycated end products (AGEs), formation of superoxide radical, as well as increase in protein glycosylation, inflammatory mediators and glucose autoxidation (Garay-Sevilla et al., 2005; Toma et al., 2012; Matsuda & Shimomura, 2013).

Consequently, when overproduction of ROS occurs, this surpasses the antioxidant system's capacity to counterbalance and eliminate these species, subsequently resulting in oxidative stress (Rahman et al., 2012; Sies, 1986). The oxidative stress in the diabetic state is due to autoxidation of glucose level which usually leads to free radical generation and disruption of cellular homeostasis (Khan et al., 2015).

2.2 Streptozotocin and diabetic induction

Streptozotocin (STZ) has been used for the induction of diabetes in animal models (Szkudelski, 2001; Lenzen, 2008) which is characterised by hyperglycemia and disruption of the cellular component of vital tissues. It has a molecular formula of $C_8H_{15}N_3O_7$ and a molecular weight of 265 g/mol. The structure comprises of a nitrosourea moiety with a glucose molecule attached at one end and a methyl group at the other end (Figure 1).

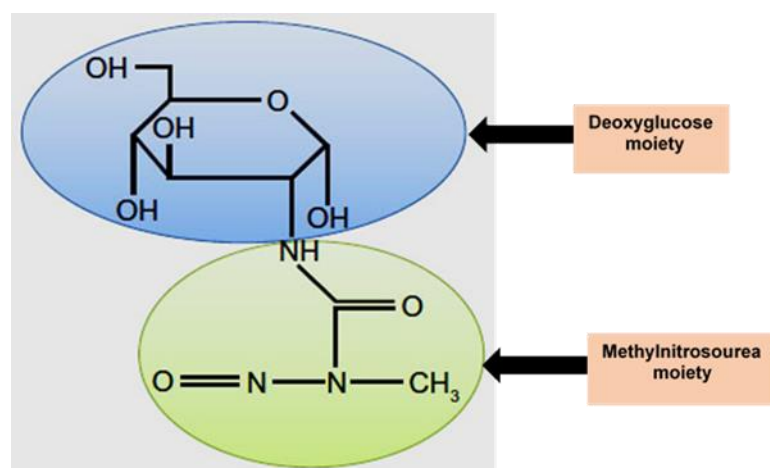


Figure 1: Structure of Streptozotocin (Reproduced from Wu and Yan, 2015)

It is a cytotoxic drug, selectively exerting its toxic effect on the β -cells of the pancreatic islet, destroying them and inducing impairment of the islet via glucose oxidation (Akbarzadeh et al., 2007). The glucose moiety allows preferential uptake of STZ into β -cells, probably via the glucose transporter 2 (GLUT-2) receptors, making β -cells specific targets of STZ (Vetere et al., 2014). Intracellular metabolism of STZ generates ROS and nitric oxide giving rise to further DNA fragmentation and a sequence of oxidative reaction leading to cell death (Figure 2).

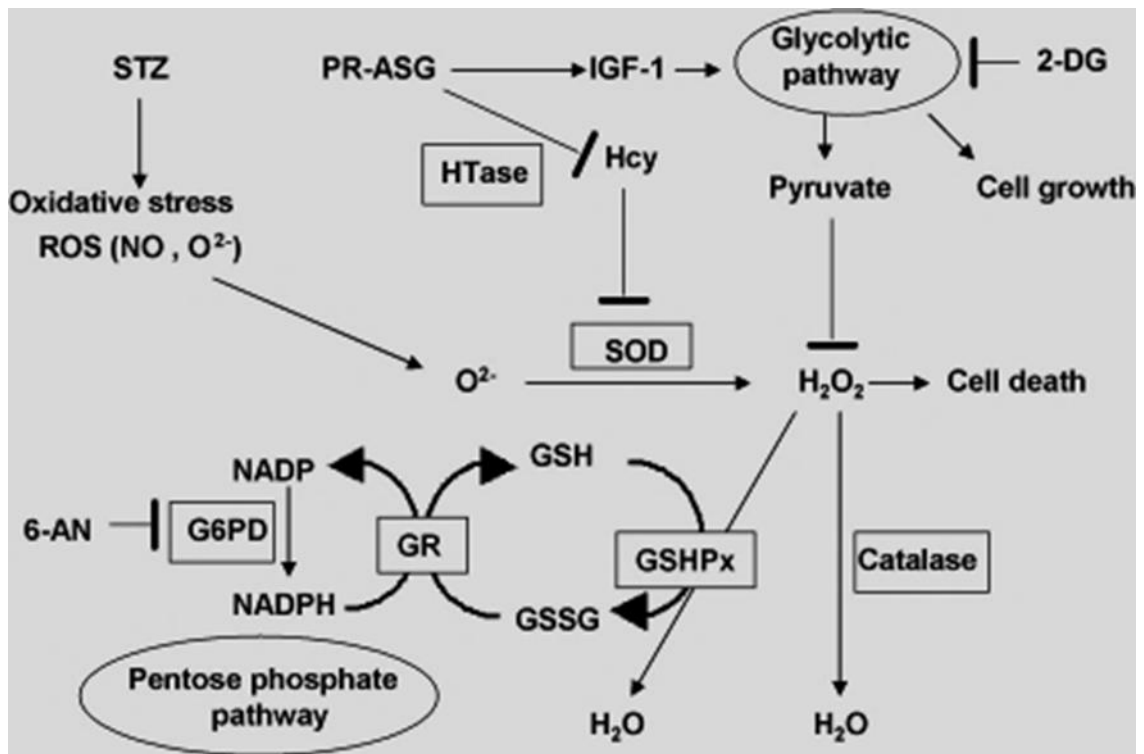


Figure 2: Mechanism of action of streptozotocin (Reproduced from Usuki et al., 2011)

2.3 Classification of diabetes

There are typically three main classifications of DM; type 1, type 2 and gestational diabetes.

Type 1- is an autoimmune disease which destroys insulin producing pancreatic cells, whereby no insulin is secreted as a result of pancreatic β -cell deterioration and reliance on exogenous insulin for survival (Alberti et al., 1998). About 10% of all diabetics are type 1 with dependence on insulin for survival with risk of ketoacidosis (American Diabetes Association, 2010). Since this type is auto-immune, a preventive regime is yet to be known.

Type 2- is a progressive disease typified by insufficient production of insulin or insulin resistance (Franz, 2012; Mohamed et al., 2016a). About 90% of all diabetes incidences are type 2 and it is the second highest risk factor for developing Alzheimer's disease (Breteler, 2000; CDCP, 2011). Oxidative stress has been implicated in the pathology of type 2 diabetes (Evans et al., 2002; Giacco & Brownlee, 2010).

Gestational diabetes- is most common in pregnant women. It is characterized by a rise in glucose level and insufficient insulin which reduces glucose level (Metzger et al., 2007). A large number of individuals develop pre diabetes before a diabetic condition is diagnosed. In

prediabetes, the glucose level is consistently above normal and often progresses to type 2 diabetes. Gestational diabetes is characterized by a slight resistance to insulin (Metzger et al., 2007).

Monogenic diabetes has been newly diagnosed which is characterised by a single gene mutation in the mitochondrial DNA or the autosomal dominant inheritance pattern. It is most common in young individuals (Buchanan & Xiang, 2005; Yang & Chan, 2016).

2.4 Diabetic symptoms and complications

Diabetic symptoms include excessive thirst (polydipsia), excessive weight gain or loss, blurred vision, excessive hunger (polyphagia), fatigue, frequent infections, frequent urination (polyuria), muscle loss, dehydration, slow healing of sores and wounds, trembling, depression, dizziness, erectile dysfunction, and numbness (Ciechanowski et al., 2003; Genuth et al., 2003). There are chronic diabetic complications arising from hyperglycemia which could possibly lead to glucotoxicity causing damaging effects to various cells in the body (Wu & Yan, 2015). These damaging effects are associated with pancreatitis, diabetic retinopathy, nephropathy, hepatopathy and neuropathy (Matsuda & Shimomura, 2013). Other complications implicated in diabetes include stroke, coronary and peripheral arterial diseases (Brownlee, 2001).

Diabetic Pancreatitis

Pancreatitis occurs when the pancreas is inflamed. Acute pancreatitis is a known complication of diabetes, although there are no studies linking diabetes as a causative of acute pancreatitis (Blomgren et al., 2002). The pancreas synthesizes digestive enzymes and hormones that help regulate glucose and metabolism of fats and protein (Mahadevan, 2016). Glucagon and insulin are produced by the pancreas which aids in glucose regulation. In type 1 diabetes, β -cells are destroyed leading to insufficient insulin production (Lumelsky et al., 2001). Transient hyperglycaemia might be seen in individuals with pancreatitis (Khan et al., 2015).

Studies revealed that individuals with type 2 diabetes may be at higher risk of acute pancreatitis and biliary disease than individuals without diabetes (Noel et al., 2009; Girman et al., 2010). Severe acute pancreatitis results in low blood pressure, dehydration and can cause failure of vital organs (Mahadevan, 2016).

Diabetic Neuropathy

Diabetes is a major cause of peripheral neuropathy, with about 30% of individuals with diabetes developing neuropathy (Lindsay et al., 2010). Diabetic neuropathy occurs when there is damage to the nerves of the peripheral nervous system resulting in severe diabetic complications (Callaghan et al., 2012). In diabetic conditions, the walls of the tiny blood vessels responsible for the supply of nutrients and oxygen to the nerves in organs, hands and feet are damaged (Sen et al., 2016). Diabetic neuropathy is associated with hyperglycemia, low insulin production, distal symmetrical polyneuropathy and mechanical injury to the nerves (Vinik et al., 2013). Current reports have indicated diabetic neuropathy as the most common complications of diabetes pose a high health risk globally (Jack & Wright, 2012, Hinder et al., 2013).

Diabetic Retinopathy and Nephropathy

Diabetes contributes to an increase in oxidative stress, which subsequently plays a pivotal role in the pathogenesis of diverse diabetic complications (Feldmen, 2003; Brownlee, 2005; Kowluru & Kanwar, 2009). Diabetic effect on structural proteins of the retina leading to diabetic retinopathy, cataracts and glaucoma have been documented (Feldman, 2003; Ahmad et al., 2009). Enduring cycle of metabolic stress, damage of tissue and cell death may amplify the oxidative stress, nitrate stress and lipid peroxidation status resulting in increased damage to DNA and free radicals production (Kowluru, 2003).

Diabetic nephropathy is a life-threatening complication of type 1 diabetes and continues to increase worldwide (Chakraborty et al., 2012). It causes progressive damage to the capillaries of the kidney's glomeruli leading to endothelial dysfunction, changes in inflammatory

biomarkers and oxidative stress (Toma et al., 2012; Forbes & Cooper 2013). Diabetes nephropathy has been investigated in diabetic individuals revealing elevated level of microalbuminuria which precedes the development of diabetic nephropathy (Marketou et al., 2016). It is characterised by the presence of micro-albuminuria with a low abnormal level of albumin in the urine which could lead to further kidney damage, cardiovascular morbidity and eventually mortality (Huang et al., 2002; Navarro-González et al., 2016).

Diabetic Hepatopathy

The liver plays a vital role in the regulation of carbohydrate metabolism. It supplies glucose to other organs that require glucose as an energy source and is one of the organs affected by diabetes. In the diabetic state, there is an increased generation of ROS which triggers a chain of reaction leading to the peroxidation of lipids, lipoprotein modifications and several cellular mutations of biomolecules (Miller et al, 2011; Mbikay, 2012). This peroxidation leads to oxidative stress which is an important risk factor in the pathogenesis of cardiovascular and chronic diseases such as diabetic and associated complications (Forbes & Cooper, 2013).

Moreover, there is increasing evidence from clinical studies showing oxidative stress as a major player in diabetic pathophysiology leading to dyslipidemia, impaired glucose tolerance, β -cell dysfunction, and ultimately resulting in liver malfunction (Ghosh et al., 2015; Tangvarasittichai, 2015). Diabetic hepatopathy is linked to liver failure and liver cirrhosis. Glycogenic hepatopathy has been reported in other studies conducted which show pathological overloading of the hepatocytes with glycogen and transaminase (Torbenson et al., 2006). Torbenson (2006) also diagnosed hepatomegaly in individuals with poorly controlled type 1 diabetes.

Cardiovascular diseases

Cardiovascular diseases (CVD) is the number one causative of mortality globally; with diabetic individuals at higher risk (Beulens et al., 2010). Oxidative stress is involved in the onset and progression of diabetes (Matough et al., 2012), leading to cardiovascular complication,

ischemic heart disease and myocardial dysfunction (Patel et al., 2012b; Mbikay, 2012; Ezuruike & Prieto, 2014). The low antioxidant defence as a result of diabetic complications may contribute to cardiovascular disease, nerve damage, and nephropathy (He & King, 2004). Therapeutic interventions are currently been employed in the prevention and treatment of cardiovascular diseases (Rader & Hovingh, 2014).

2.5 Reactive oxygen species and oxidative stress in diabetes

Oxidative stress occurs in a biological system when there is over production of ROS and deficiency in enzymatic or non-enzymatic antioxidant (Rahman et al., 2012). Oxygen is essential for metabolic processes to take place. However, oxygen is the main source of free radicals. Many elements comprise molecules which have electrons in their outer orbital. Chemical bonds are formed between these elements dissociate during oxidation such as oxidative phosphorylation in mitochondria resulting in highly reactive species with unpaired electrons in their outermost orbital. Molecular oxygen is reduced by one electron during oxidation to form superoxide radical ($O_2^{\cdot-}$) a precursor for most ROS. Superoxide radical further reacts with an electron to form hydrogen peroxide (H_2O_2). Hydrogen peroxide formed can be partially reduced to hydroxyl anion (OH^{\cdot}) or fully reduced to water. ROS are either free radicals, e.g. hydroxyl radical (OH^{\cdot}), peroxy radical, superoxide radical ($O_2^{\cdot-}$), ozone (O_3), or non-radical reactive compounds such as singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2) (Tan et al., 2007; Halliwell & Gutteridge, 2015). Free radicals are atoms or molecules with lone pair of electrons and are capable of independent existence (Murdolo et al., 2013). Free radicals have been implicated in a number of disease conditions including diabetes (Murdolo et al., 2013).

Reactive nitrogen species (RNS) are molecules derived from nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) such as nitroxyl anion, nitrosyl iron complexes, nitrosonium cation, peroxynitrite and S-nitrosothiols all of which play vital roles in the physiological regulation of cells. At high concentrations, RNS is known to cause cell injury inducing nitrosative stress

(Martínez & Andriantsitohaina, 2009). Factors that promote reactive nitrogen species generation include normal metabolic processes such as aging, stress, poor diet, pollution, radiation, infection, disruption of the nitric oxide pathway and drugs as well as an inflammatory response (Zhang et al., 2002). These reactive species (hydroxyl, peroxy, superoxide radical and hydrogen peroxide) initiate cumulative damage to proteins, lipids and nucleic acids (Figure 3) and eventually lead to change in structure and function of organs and cellular components (Bhosle et al., 2005; Halliwell & Gutteridge, 2015).

Furthermore, free radicals are generated during enzymatic metabolism, inflammation, radiation and decreased nitric oxide production. These are excessively produced in diabetes during glucose oxidation, secreted by inflammatory cytokines causing an imbalance between ROS production and antioxidant defence activity, culminating in oxidative stress (Dröge, 2002; Jaiswal et al., 2013). A continuous build-up of these reactive species contributes to cellular damage, apoptosis, lipid peroxidation and eventually cell death in diabetes thus enhancing the pathogenesis of the disease.

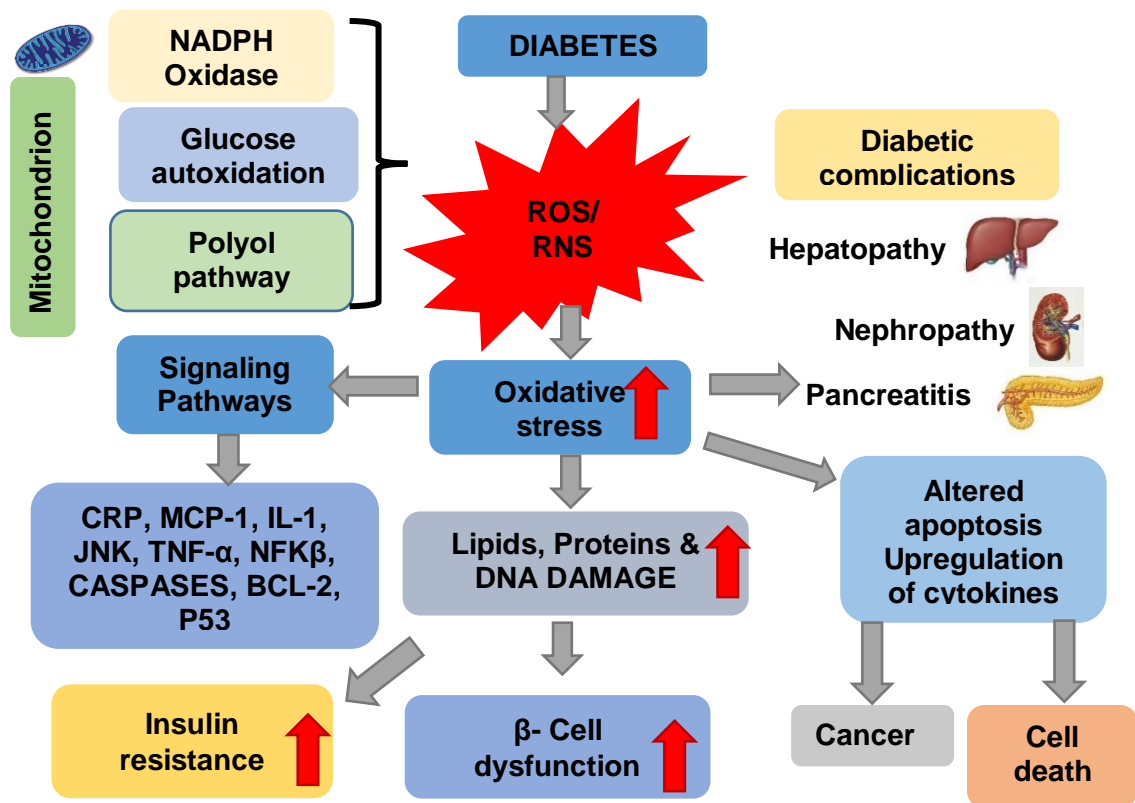


Figure 3: Relationship between oxidative stress and diabetes

2.6 Diabetes and inflammation

Chronic Inflammation is associated with diabetes and has been reported to cause severe organ damage in diabetic rats (Mohamed et al., 2016b). Individuals with diabetes have a high level of inflammatory cytokines, activation of leukocytes and increased tissue fibrosis (Donath & Shoelson, 2011). Inflammatory cytokines; C-reactive protein (CRP), interleukin-1 α (IL-1 α), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-18 (IL-18), tumour necrotic factor- α (TNF- α) and chemokine; monocyte chemotactic protein-1 (MCP-1) concentrations are high in diabetic conditions. Inflammation in tissues occurs in response to harmful stimuli or damage to cells. IL-6 and TNF- α cytokines play a crucial role in hyperglycemia-induced kidney injury and are associated with the development of diabetes. Increasing levels in patients with diabetic nephropathy have been reported, suggesting that these cytokines play significant roles in the pathogenesis of diabetic nephropathy (Navarro- González & Mora-Fernández, 2008). Seca et al. (2014) noted the presence of high concentration of inflammatory proteins which negatively impacted on the function of insulin.

2.7 Diabetes and lipid peroxidation

Lipid peroxidation results from free radical chain reactions causing deterioration in the lipids of the cell membrane; affecting the structural and physiological integrity of cells and has been reported in diabetic conditions, kidney diseases and disruption in blood vessels (Wilcox, 2002; Afshari et al., 2007). Diabetes causes disruption in lipid metabolism particularly an increased exposure to lipid peroxidation. People with types 1 and 2 DM often suffer from a vascular disease which is related to oxidative damage of membrane lipids that affects the structure or functions of vital organs resulting from free radicals generation (Murdolo et al., 2013). Antioxidants; non-enzymatic or enzymatic, help to mop up free radicals and ROS preventing lipid peroxidation thereby reducing deleterious effect caused by this species (Sena & Chandel, 2012; Murdolo et al., 2013).

2.8 Diabetes and apoptosis

Apoptosis, also known as programmed cell-death, is executed by caspases which are critical for maintaining tissue homeostasis. Apoptosis involves the prompt removal of unwanted cells for maintenance of specific organs (Nakanishi et al., 2009), and is essentially a prominent feature in some disease state including diabetes. ROS generation results in immune response inducing apoptotic cell death via membrane receptors and intracellular stress (Matés & Sánchez-Jiménez, 2000). Diabetes is also associated with the production of pro-apoptotic factors (Barclay et al., 2015). Some apoptotic markers are NFK β , p53, caspase 3, caspase 7, caspase 9 and BCL-2. Caspase 3 is an executioner caspase, with a high level seen in a diabetic state where the neural cells are lost and increases excessive cell death and it is regulated by BCL-2 (Korsmeyer, 1999; Hui et al., 2004). Fundamentally, BCL-2 is an anti-apoptotic protein that regulates cell death and promotes cell survival. It determines the commitment of cells to apoptosis (Ahmad et al., 2009; Wang, 2015).

2.9 Mechanism of action of polyol pathway and its relationship with diabetes

High glucose levels are metabolised through the polyol pathway which induces oxidative stress. The polyol pathway is also known as the sorbitol-aldose reductase pathway. Aldose reductase (AR) is able to use a range of carbonyl compounds as substrates, causing a reduction to their corresponding sugar alcohols (polyols). High concentrations of glucose in the cell is reduced to sorbitol and NADPH is oxidised to NADP⁺ (Giacco & Brownlee, 2010). NADPH is a fundamental co-factor necessary for the regeneration of a vital intracellular antioxidant; its depletion halts generation of reduced glutathione leading ROS production (Nishikawa & Araki, 2013). Sorbitol is subsequently oxidised to fructose by sorbitol dehydrogenase (SDH) and NAD⁺ is reduced to NADH in the second step of the pathway (Figure 4). NADH further generates ROS. Fructokinase phosphorylates fructose to fructose-3-phosphate and glyceraldehyde-3-phosphate 3-deoxyglucosone (3-DG) forming D-glyceraldehyde-3-phosphate, which acts as a substrate for glycolysis (Giacco & Brownlee, 2010). However, in diabetic complications, aldose reductase metabolises these unused or

excess glucose to a toxic substrate. Consequently, exposure of endothelial cells to free radicals reduces the amount of reduced glutathione and oxidative stress is increased (Brownlee, 2005).

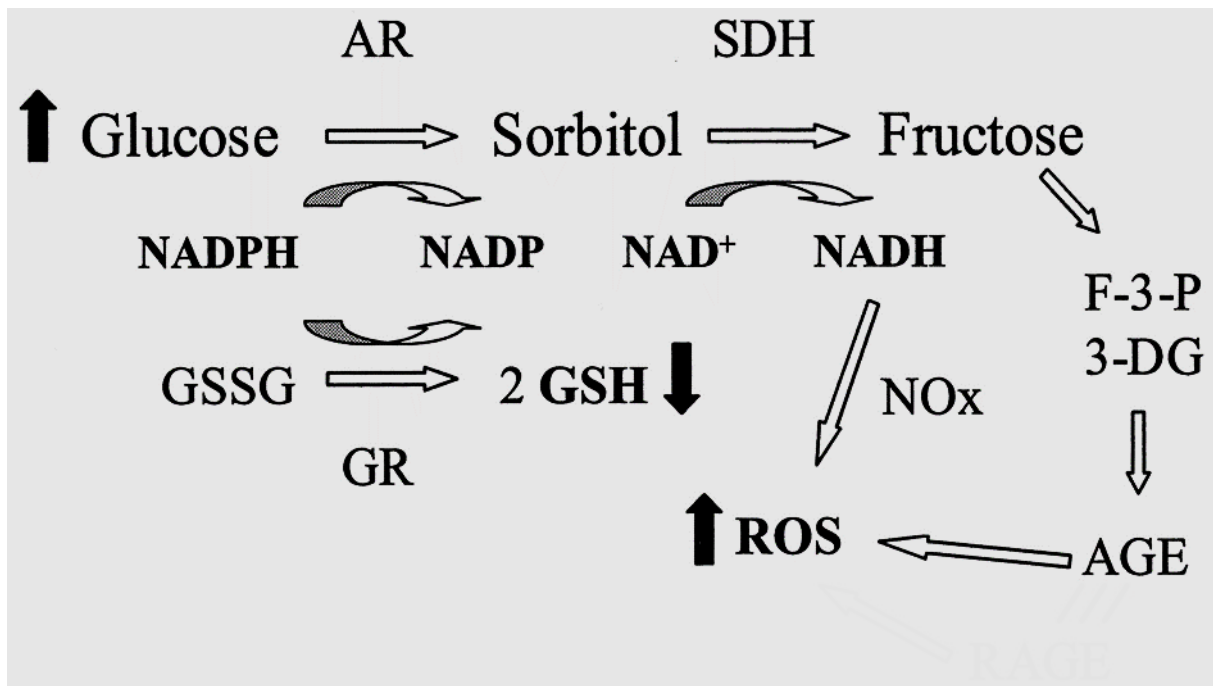


Figure 4: The polyol pathway (Reproduced from Chung et al., 2003)

2.10 Mechanism of action of hexosamine biosynthesis pathway and its relationship with diabetes

Hexosamine pathway is a path of glycolysis responsible for the detrimental effects of chronic hyperglycaemia. It has been shown that flux through the hexosamine pathway (Figure 5) may be increased by glycolysis inhibition or increased glucose entry (Buse, 2006). This also plays a significant role in the development of insulin resistance, and vascular complications of diabetes (Rossetti, 2000; James et al., 2002). Glucose-6-phosphate is converted to fructose-6-phosphate then further converted to glucosamine-6-phosphate which is catalysed by Glutamine: fructose-6-phosphate amidotransferase (GFAT) yielding Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as an end product (Figure 5). This pathway is essential for the synthesis of glycolipids and glycoproteins glycosyl side chains. UDP-GlcNAc is responsible for flux regulation in this pathway. Increased flux is implicated in β -cell dysfunction in diabetes (Buse, 2006; Fiorentino et al., 2013). Suppression of the process of glucose transport may

occur as a result of the elevated intracellular O-GlcNAc-mediated alteration of particular kinds of protein (Vaidyanathan et al., 2014).

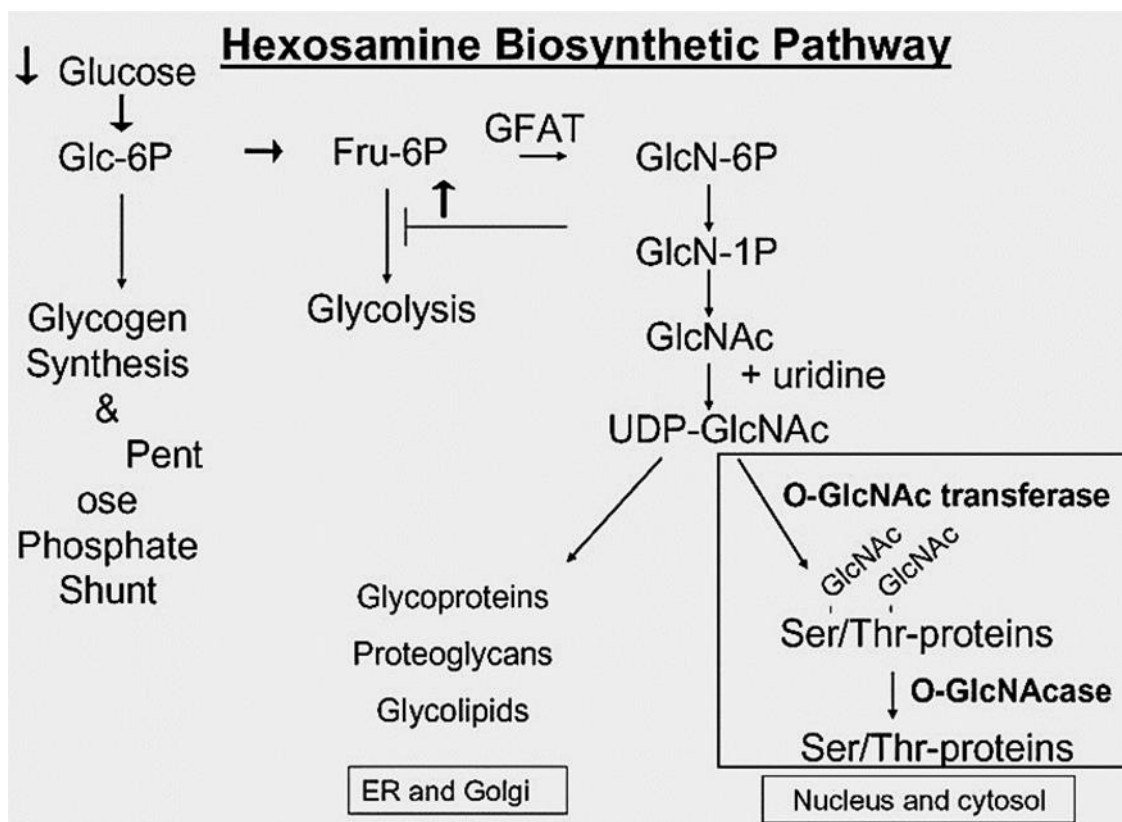


Figure 5: The hexosamine biosynthetic pathway (Reproduced from Buse, 2006)

2.11 Mechanism of action of advanced glycated end products (AGEs) formation pathway and its relationship with diabetes

Glycated proteins and lipids are irreversible AGEs and their synthesis is increased in the onset of degenerative disease such as diabetes (Garay-Sevilla et al., 2005; Goldin et al., 2006). Their formation causes macrovascular changes. Several mechanisms has been linked to the generation of AGEs with the activation of protein kinase C (PKC) isoforms and carbonyl stress pathway, where sugars or lipids are oxidised to form dicarbonyl intermediates that bind to amino acids forming AGEs (Figure 6) (Miyata et al., 1998; Huebschmann, et al., 2006).

NADPH oxidase pathway is also another pathway for AGEs generation via the receptor for advanced glycation end-products (RAGE) activation which damage the mitochondria and induces oxidative stress (He & King, 2004; Ramasamy et al., 2005). Under pathological

conditions, AGEs modifies the extracellular proteins thus activating growth factors in cytokines, while they also cause changes to biomolecule and cells resulting in vascular disease, aging and damage to the kidney with the decreased urinary output (Glenn & Stitt, 2009; Semba et al., 2009). Natural antioxidant sources have been reported to lessen the formation of AGEs, by breaking cross-links after they are formed and preventing feedback loops (Mizutani et al., 2000; Tang, 2014).

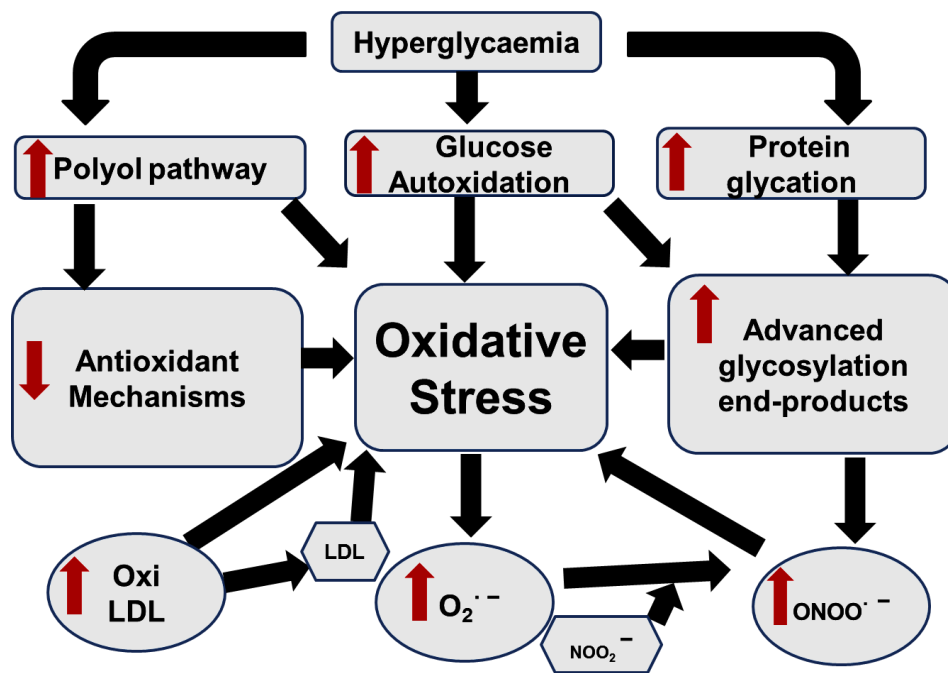


Figure 6: Hyperglycemia and activation of (AGEs) (Produced by Omodanisi, 2017)

2.12 Mechanism of action of protein kinase C (PKC) activation pathway and its relationship with diabetes

Diabetes plays a major role in the development of vascular dysfunction and pathologies (Patel et al., 2012b). PKC is of the family of serine-threonine kinases and plays a significant role in intracellular signal transduction for hormones, cytokines and gene expression. PKC contains two functional regulatory domains C1 and C2 and uses ATP to phosphorylate amino acid side chains in target proteins. PKC regulates a number of vascular functions and is activated by diacylglycerol (DAG) and phosphatidylserine.

Hyperglycemia stimulates the activation of diacylglycerol-protein kinase C (DAG-PKC) pathway (Figure 7) and can cause the alterations in other enzymatic activity, gene expression

and leads to oxidative stress: resulting in multiple functional changes in vascular tissues (Singh et al., 2014). When the metabolic pathways are activated, oxidative stress and diabetes induce cell damage resulting to diabetic neuropathy. Understanding PKC activation and inhibition in diabetes will help facilitate therapeutic remedies.

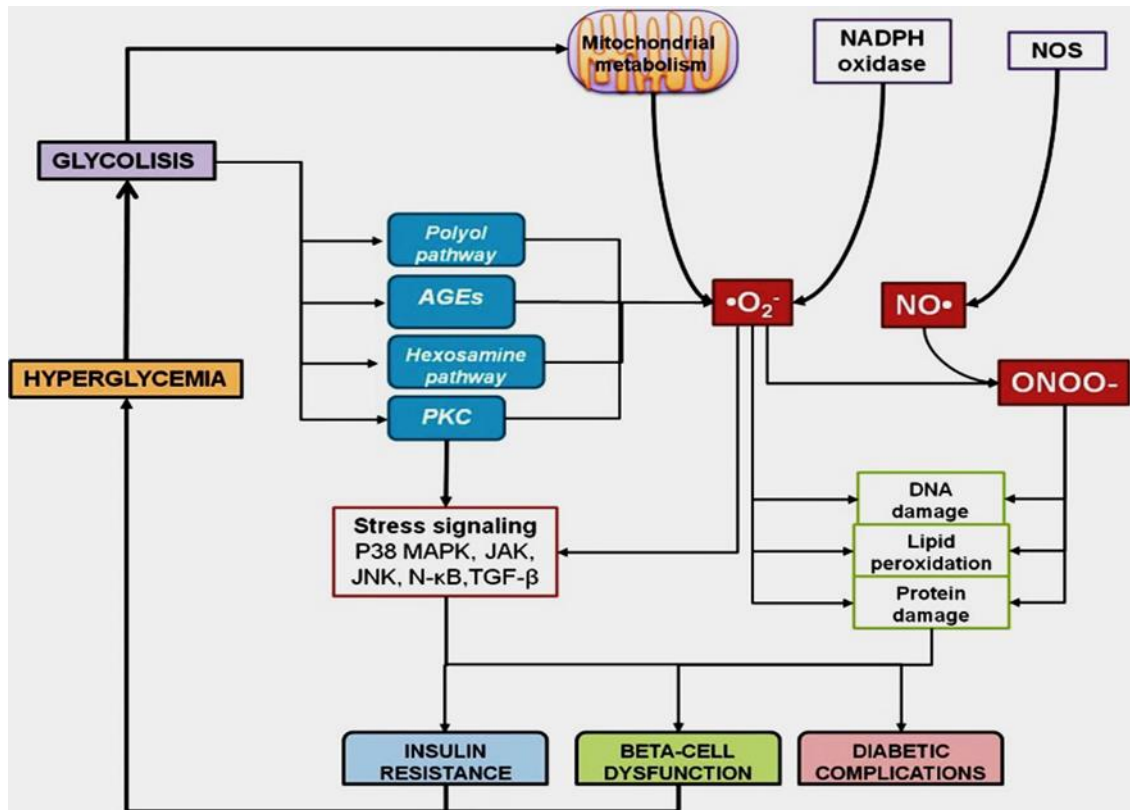


Figure 7: Hyperglycaemic activation of metabolic pathways (reproduced from Lazo-de-la-Vega-Monroy & Fernandez-Majia, 2013)

2.13 Prevention of diabetes

Individuals with type 1 diabetes are advised to have a healthy and balanced eating plan, exercise regularly and take insulin (American Diabetes Association, 2004). For patients with type 2 diabetes, it is imperative to have a balanced and healthy eating plan. Physical activity, oral medication and sometimes insulin injection are also required to manage blood glucose levels (Jara et al., 2011). Individuals with both types of diabetes must carry out regular blood tests.

Overweight and obese individuals are predisposed to developing type 2 diabetes (Chen et al., 2012). As a preventive measure against diabetes, engaging in regular exercise will help maintain normal growth and development, improve tissue sensitivity to insulin and keep the body physically fit. Proper diet with a balanced ratio of nutrients, eating plenty of fruits and fresh vegetables including *Moringa oleifera*, are important components for comprehensive weight management strategy. An increase in aerobic exercise will result in a decreased risk of developing heart disease (Goran et al., 2003).

2.14 Treatment of diabetes

Transplantation of the pancreas via surgery is usually an option for individuals with severe type 1 diabetes (Efrat, 2002; Robertson, 2004; American Diabetes Association, 2014). For a significant number of patients, type 2 diabetes can be reversed through gastric bypass surgery, although early intervention is usually a better option to prevent reoccurrence (Schauer et al., 2003; Dixon et al., 2008).

In addition to insulin, many anti-diabetic drugs are also in use and these include sulfonylurea (glimepiride, Glipizide), alpha-glucosidase inhibitors (acarbose), dipeptidyl peptidase IV inhibitors (sitagliptin), meglitinides (nateglinide), biguanides (metformin), and thiazolidinediones (pioglitazone) (Nathan, 2007; Bosi, 2009). However, owing to their numerous side effects which include abdominal bloating, hypoglycaemia (a serious adverse effect), gastrointestinal (GI) disorder, liver injury, weight gain and hypersensitivity reactions, their use is limited (Del et al., 2007). Plants have helped in improving the health status of many with appreciable results and still positively contribute to the treatment and management of diseases (Dutta et al., 2008). The rapid discovery of various medicinal plants and natural products with anti-diabetic potentials has provided a remarkable intervention in the history of many diseases including diabetes (Al-hallaq et al., 2013).

Due to the numerous health benefits of MO, further studies to investigate its potential effect in the treatment and management of diabetes using an animal model in a more comprehensive and extensive depth could potentially revolutionize new drug discovery for diabetic patients (Farooq et al., 2012).

2.15 Antioxidant biomolecules

Antioxidants are chemicals or biological agents capable of neutralizing the potentially damaging action of free radicals. In the diabetic state, free radicals increase can be mopped up by antioxidants. The body system has a well-developed antioxidant defence mechanism that helps to prevent and scavenge free radicals formation thereby limiting their deleterious effects (Pham-Huy et al., 2008). Also, antioxidants act by giving up electrons to free radicals making them stable and preventing their cascade of reaction. They further proffer a protective effect to cells, minimising the destructive effect of free radicals. Antioxidants defence mainly act via these mechanisms; prevent the formation of free radicals, scavenge radicals formed or repair damages caused by oxidative stress (Kim et al., 2009).

2.15.1 Enzymatic antioxidants

Superoxide dismutase (SOD) readily converts highly reactive superoxide radical to a less reactive hydrogen peroxide (H_2O_2) in order to maintain an optimal cellular function (Speisky et al., 2009). Catalase (CAT) acts by breaking down H_2O_2 formed in cells to molecular oxygen and water (Winterbourn, 2014). Glutathione peroxidase (GPx) is a selenocysteine-containing enzyme and it functions in scavenging and removing hydrogen and lipid peroxide from cells. It does this in the presence of reduced GSH and NADPH. GPx reduces H_2O_2 and organic peroxide to water and alcohol thereby inhibiting the formation of free radicals (Rani & Yadav, 2015).

Reduced glutathione (GSH) is a tripeptide with a rare peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine (Castro et al., 2008). It is

the primary antioxidant in liver cells which detoxifies radicals thereby defending cells against damage while GSH helps in detoxification and defence against free radicals and cytotoxins (Halliwell, 2011).

2.15.2 Non-enzymatic antioxidants

Exogenous antioxidant sources include vitamin C, E, A, carotenoids, phenols and glucosinolates which protect cells from free radicals generation and stabilizing radicals (Krishnamurthy & Ashish, 2012; Schieber & Chandel, 2014). Presently, there has been appreciable interest in various therapeutic remedies to disease conditions with minimal or negligible side effects (Al-Snafi, 2015). In recent times, medicinal plants including MO have attracted the attention of researchers for their therapeutic and phytochemical properties in treating and managing various diseases including DM (Sreelatha & Padma, 2009; Kumari, 2010; Wang et al., 2012; Jung, 2014).

2.16 Medicinal Plants

Despite the presence of many methods, approaches and therapies, the management of DM remain unsatisfactory (Singh et al., 2001). The increasing prevalence of diabetes in both developed and developing countries has challenged scientists to further explore various therapeutic agents that can be used in the treatment and management of disease conditions possibly more efficiently (Gupta et al., 2012). Antidiabetic drugs are expensive and not affordable for low socioeconomic individuals, thus the need to explore other inexpensive herbal sources for the treatment of diabetes (Singh et al., 2001; Amod et al., 2012).

Plants have played vital roles in the maintenance of human health and the improvement of the quality of life of people, serving as important components for medicines, beverages and seasonings (Elder, 2004). Interestingly, many indigenous plants contain phytochemicals that exhibit both anti-diabetic and antioxidant properties (Sánchez et al., 2006; Dièye et al., 2008).

2.16.1 *Moringa oleifera*

The rapid discovery of various medicinal plants and natural products with anti-diabetic and hypoglycemic potentials has provided a remarkable intervention in the history of various diseases including diabetes (Umashanker & Shruti, 2011; Al-hallaq et al., 2013). Ayurvedic medicine uses plants to promote self-healing, to attain good health, and achieve longevity. MO can provide therapeutic constituent to prevent and treat many disease conditions (Singh et al., 2009). MO is native to Northwest India and has its origin in Agra and Oudh, South of the Himalayan Mountains (Mishra et al., 2011). The plant is now distributed worldwide in Africa, Asia, South and Central America and the Caribbean.

MO belongs to the family of Moringaceae (Sreelatha et al., 2011) and known by several names in various countries where it is used as Miracle Tree, Horseradish, Drumstick, Benzolive tree, *Saijan*, *Munaga*, *Ewe-ile*, *Zogale*, *Odudu Oyingbo*, *Sajna*, *Kamungay*, *Sohanjina*, *Mulangay*, *Mupulanga*, *Marango*, *Mlonge*, *Saijhan*, and *Shevaga* (Fahey & Sc 2005; Muhammad & Soriani, 2014). MO has been in existence for centuries, dating as far back as early 2000 BC and it is a very valuable plant because of its vast medicinal properties. There are twelve other species of *Moringa* but *Moringa oleifera* (Figure 8 a & b) is the most common and researched of all species. Other species are *Moringa stenopetala*, *Moringa ruspoliana*, *Moringa rivae*, *Moringa pygmaea*, *Moringa peregrine*, *Moringa ovalifolia*, *Moringa longituba*, *Moringa hildebrandtii*, *Moringa drouhardii*, *Moringa concanensis*, *Moringa borziana* and *Moringa arborea* (Atawodi et al., 2010).



Figure 8: *Moringa oleifera* leaf (a) seeds with husk (b)

2.16.2 Uses of *Moringa oleifera*

Essentially, all parts of MO such as the leaves, flowers, root, bark, seeds and gum have numerous uses (Anwar et al., 2007; Kumar et al., 2010). MO is an edible plant (young leaves) which can be eaten as a vegetable and used as beverages. Various parts of MO such as the leaves (Figure 8a), seeds (Figure 8b), roots, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, while they also possess antitumor, antiulcer, antipyretic, antiepileptic activities (Farooq et al., 2012). Further, MO has antimicrobial, antifungal, anti-tuberculosis, analgesic, anti-cancer, anti-diabetic, antispasmodic, diuretic and antihypertensive activities (Shanker et al., 2007; Singh et al., 2009; Kumar et al., 2010; Prod et al., 2012).

MO has been found to show hypolipidaemic, and antiatherosclerotic effect (Faizi et al., 1994; Iqbal & Bhanger, 2006; Chumark et al., 2008). MO is used in alley cropping (biomass production), domestic cleaning agent (crushed leaves), animal forage (leaves and treated seed-cake), blue dye (wood), biogas (from leaves), foliar nutrient (juice extracted from the leaves), fertilizer (seed-cake), green manure (from leaves), honey (flower nectar) honey and sugar cane juice-clarifier (powdered seeds), gum (from tree trunks), ornamental plantings, tannin for tanning hides (bark and gum), pulp (wood), bio pesticide (soil absorption of leaves to forestall seedling damping off) and water purification (Babu & Chaudhuri, 2005; Fahey & Sc 2005). MO seed oil, also called ben oil, has about 30-40 % yield by weight and it is a sweet, non-drying and non-sticking oil that wards off rancidity (Mahajan et al., 2009).

It is interesting to note that this plant can also grow in regions where the soil is not very fertile and is even capable of being grown in garden space at home. MO grows well in sandy soil with a slightly acidic pH of 6.2 to 7.0 and has a height ranging from 5-12 m with a straight trunk 10 - 30 cm thick (Hussain et al., 2014).

2.16.3 Phytochemicals in *Moringa oleifera*

Studies on the phytochemical properties of MO by Ndong et al. (2007) reported that MO is rich in phenols such as kaempferol glycosides, rutin, chlorogenic acids and quercetin glucosides. Carotenoids such as B-carotene, lutein, xanthin, zeaxanthin, luteoxanthin have been reported to be present in the leaves of MO (Pullakhandam & Failla, 2007; Saini et al., 2014).

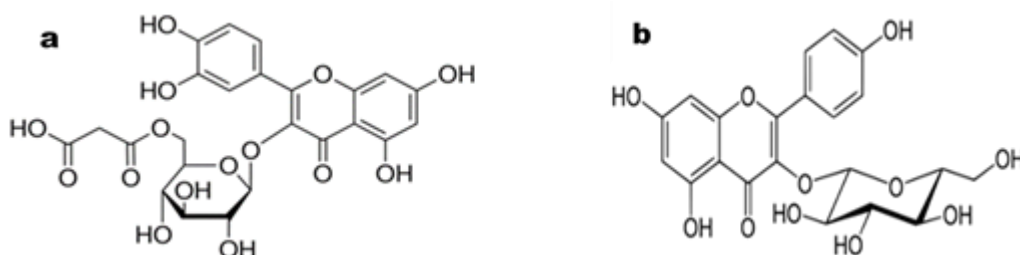


Figure 9: Phenols in *Moringa oleifera* (a) Quercetin 3-(6''-malonyl-glucoside) and (b) Kaempferol-3-O-glucoside

Some active compounds such as flavonol-glycosides, benzaldehyde 4-O-beta-glucoside and benzoic acid 4-O-beta-glucoside were isolated and characterized from the methanol extract of MO leaves (Bennett et al., 2003; Manguro & Lemmen, 2007). Qualitative analysis carried out on the leaves revealed the presence of phenolic compounds such as quercetin 3-(6''-malonyl-glucoside) (Figure 9a), quercetin-3-O-glucoside, kaempferol-3-O-glucoside also known as Astragalalin (Figure 9b), flavonoids, anthocyanins, proanthocyanidin, cinnamates and trace amount of alkaloid, 3-caffeoylquinic and 5-caffeoylquinic acid (Sreelatha et al., 2011).

The leaves of MO contain phytochemicals such as niazirin and niazirin (Goyal et al., 2007). Water soluble polysaccharides such as d-galactose, 6-O-Me-D-galactose, D-galacturonic acid, l-arabinose and l-rhamnose in a molar ratio of 1:1:1:1 were isolated from the aqueous extract of pods from MO (Roy et al., 2007). MO contains a high concentration of vitamins A, B and C, minerals (especially iron) and amino acids such as leucine, glutamic, valine, aspartic and alanine (Table 1). Also, the seed of MO was reported to contain various sterols, tocopherols and fatty acids from the n-hexane extract (Ruttarattanamongkol et al., 2014).

Table 1: Amino acids content of *Moringa oleifera* leaves

	Fresh Leaves	Dried Leaves
Arginine	406.6 mg	1.325 mg
Histidine	149.8 mg	613 mg
Isoleucine	299.6 mg	825 mg
Leucine	492.2 mg	1.950 mg
Lysine	342.4 mg	1.325 mg
Methionine	117.7 mg	350 mg
Phenylalanine	310.3 mg	1.388 mg
Threonine	117.7 mg	1.188 mg
Tryptophan	107 mg	425 mg
Valine	374.5 mg	1.063 mg

All values are per 100 grams leaves of edible portion (reproduced from Gupta et al., 2012)

2.16.4 Therapeutic potentials of *Moringa oleifera*

MO is well known traditionally for the treatment of DM, hepatotoxicity, rheumatism, venomous bites and also for cardiac stimulation (Soliman, 2013). Individuals in developing countries, especially in Africa have used MO to treat and manage the symptoms of diabetes for several years. *Moringa* has been used for centuries as a folk remedy for catarrh, gastric ulcers, cancer, nervous conditions, headaches, hay fever, stomach complaints, increasing bone density, cramps, skin diseases, increase lactation, fatigue, oedema, impotence, haemorrhoids and sore gum (Chumark et al., 2008).

It is used as a blood cleanser and blood builder helps in wound healing and boosts the immune system (Rathi et al., 2006; Mehta et al., 2011). Also, *Moringa* leaves have bioactive constituent (β -Sitosterol) with cholesterol lowering effect. This compound is well suited for the reduction of the levels of cholesterol from the serum of high fat diet-fed rats (Farooq et al., 2012). Mehta et al. (2003) reported that the fruits of MO were found to have hypolipidaemic effect; reduced the serum cholesterol, triglyceride, phospholipid, LDL, VLDL, atherogenic index and cholesterol to phospholipid ratio in hyper-cholesterolaemic rabbits, but led to a rise in the HDL ratio (HDL/ HDL-total cholesterol) when compared with the control groups.

2.16.5 Anti-diabetic activity of *Moringa oleifera*

Various types of medicinal plants have been assessed for their potentials as beneficial agents in the treatment and management of diabetes. MO is a prominent member in this category. The leaves significantly decreased the concentration of blood glucose in Wistar rats and Goto-Kakizaki (GK) rats, model type 2 diabetes. MO is well-known for its numerous pharmacological and antioxidant action. It has been used in Bangladesh as an anti-diabetic plant which can be rationalized by the presence of active compounds (Fahim et al., 2012; Manohar et al., 2012).

In studies undertaken in India to ascertain the hypoglycemic and anti-hyperglycemic outcome of MO aqueous extract in normal (normoglycemic) and alloxan-induced diabetic rabbits respectively. It was discovered that the aqueous extract of the leaves demonstrated hypoglycemic and anti-hyperglycemic activities (Manohar et al., 2012). Jaiswal (2009) assessed the effect of the aqueous leaf extracts on glucose levels, haemoglobin, total protein, urine protein, urine sugar and body weight using three (3) different doses; 100, 200 and 300 mg/kg. The result indicated that the aqueous extract of MO leaves has hypoglycemic and anti-diabetic potentials. Also, the study elucidated the promising potential of MO, evident in the management and treatment of diabetes with minimal side effects. MO has also been found to have glucose lowering effect in STZ- induced diabetic rats perhaps through the stimulation of the β -cells of the islets of Langerhans or as a result of its insulin-like activity (Tende et al., 2011).

2.16.6 Antimicrobial and antifungal activities of *Moringa oleifera*

Currently, available therapies for diabetes management include among others, insulin injection, various oral anti-diabetic drugs and transplantation of the pancreas. Many of these, however, have several severe, adverse effects, hence, the need to consider possible safer hypoglycemic agents such as MO. A major mechanism in forestalling damage by oxidative stress is the balance of ROS and antioxidants, thus requiring the utilisation of dietary

supplementation of antioxidants-rich plants such as MO which could be a promising approach in the treatment of diabetes (Shanker et al., 2007).

MO extract is reported to cause a reduction in the serum levels of glucose and glycosylated protein in diabetic conditions while showing observable improvements in impaired glucose metabolism (Kumar et al., 2010). Its beneficial effects in various pathological conditions through its anti-inflammatory and anti-oxidative properties have been reported. However, there is a need to explore in detail its potentials in diabetic conditions (Adeyi & Nneji, 2015). MO plant has been used in folk medicine for diabetes therapeutics as well as in the treatment of other diseases (Dièye et al., 2008). Interestingly, many indigenous plants such as *Vernonia amygdalina* (VA), *Kolaviron* (Ayepola et al., 2014), contain flavonoids, glycosides, alkaloids, terpenoids, and carotenoids and have been shown to have both anti-diabetic and antioxidant activities (Patel et al., 2012a).

Antimicrobial activity of ethanolic extract of MO flowers, leaves and seeds were investigated by Renita et al. (2009). The study was carried out using micro-organisms; *Escherichia coli*, *Salmonella typhi* A *Klebsiella pneumoniae*, *Candida albicans*, *Enterobacter spp*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Staphylococcus aureus*. The treatment with the leaves led to a considerable reduction in the growth of these organisms (Renita et al., 2009). Steam distillate of MO was tested on fungal and bacterial species by Kekuda et al. (2010). It was observed that the growth of fungi was inhibited by a decrease in colony diameter in plates poisoned with distillate in contrast to control plates. Results from the study indicated more inhibition of *E. coli* compared to *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*.

2.16.7 Hepatoprotective activity of *Moringa oleifera*

The hepato-protective action of MO against acetaminophen-induced liver injury in Sprague-Dawley rats has been reported using silymarin as a standard drug. The activity was studied and a significant reduction of the levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) between groups treated with

acetaminophen alone and groups pretreated with MO were reported. Evidently, MO treated animals had glutathione (GSH) concentration restored when compared with the groups treated with acetaminophen alone (Fakurazi et al., 2008).

Hepatoprotective effect of MO seed extract was evaluated on liver fibrosis, which was induced orally by administering 20 % carbon tetrachloride (CCL₄) and MO seed extract (1 g/kg/day) simultaneously. Liver fibrosis is the over-accumulation of extracellular matrix proteins incorporating collagen that ensues in the majority of chronic liver diseases (Bataller & Brenner, 2005). The CCL₄-induced elevation of serum aminotransferase activities and globulin levels was reduced as a result of the administration of MO seed extract. Treatment with MO also results in the reduction of the elevations of myeloperoxidase activity and hepatic hydroxyproline content (Hamza, 2010).

2.16.8 Antioxidant activity of *Moringa oleifera*

Antioxidants are chemicals derived from plant, vitamins and other nutrients which guard the cells of the body system from damaging effects caused by free radicals, thus acting as a free radical scavenger (Halliwell, 2011). They inhibit the oxidation of other molecules (McCune & John, 2002). *Moringa* is a potent source of antioxidants; the leaves are high in antioxidants such as zeatin, kaempferol, quercetin, isoquercetin, rutin, β -carotene, and ascorbic acid (Anwar et al., 2007; Sikder et al., 2013) which are responsible for its ability to scavenge free radicals. (Siddhuraju & Becker, 2003). Antioxidant content of MO can be used to ameliorate oxidative stress-induced damage in association with diabetic individuals (Sreelatha et al., 2011). Experimental studies showed that methanol (80 %) and ethanol (70 %) are best solvents for the extraction of antioxidant compounds from *Moringa* leaves (Sreelatha et al., 2011).

2.16.9 Anticancer activity of *Moringa oleifera*

MO has been reported by researchers to contain anti-cancer properties. The extracts were tested using a brine shrimp lethality assay and hemolysis assay to demonstrate the anticancer

activity (Costa-Lotufo et al., 2005). The cytotoxic effects were studied on human multiple myeloma cell lines using extracts of MO leaves, results showed the least viability at the highest dose (Parvathy & Umamaheshwari, 2007).

Consequently, with the increasing rate of individuals suffering from diabetes, there is a need to investigate in more details the antioxidant, anti-inflammatory, hyperlipidaemia and anti-diabetic activities of MO in experimental animals.

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CHAPTER THREE

3 Phytochemical and antioxidant properties of different extracts of *Moringa oleifera* leaves

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ABSTRACT

Background: Antioxidant and phytochemical content of *Moringa oleifera* tree (MO) can scavenge reactive oxygen species thereby reducing and ameliorating oxidative stress associated with diabetes. In this study, we quantified the antioxidant and plant phytochemicals present in aqueous, methanol and petroleum ether extract of *Moringa oleifera* leaves.

Methods: Antioxidant potency/capacity and phytochemical content of MO extract were estimated in three extraction solvents: aqueous, methanol and petroleum ether to elucidate the various chemical constituents present in the leaves. Specific phenols and flavonoids content were identified in the methanol extract of MO using an Agilent Technology HPLC 1200 series.

Results: Results showed that the methanol extract of *Moringa oleifera* demonstrated a high antioxidant capacity and phenolic content compared to petroleum ether extract. The methanol extract revealed more chemical constituents than the petroleum ether extract. The HPLC analysis indicated the presence of flavonoids: quercetin, rutin and myricetin and phenolic acids: chlorogenic acid, sinapic acid, caffeic acid, coumaric acid, ferulic acid, gallic acid, cinnamic acid. High levels of total polyphenols, flavonols and alkaloids were reported.

Conclusion: The high phytochemical and antioxidant content of MO leaves may be responsible for its medicinal, therapeutic and pharmacological activities that offer beneficial effects against numerous diseases at low cost. The study deduces *Moringa oleifera's* capability to prevent the oxidation of other molecules by trapping free radicals and reducing the production of reactive oxygen species and inflammatory cytokines as a result of its high phenolic contents.

Keywords: *Moringa oleifera*, aqueous, methanol, petroleum ether, extract, phytochemical, antioxidant.

3.1 Introduction

MO is a widely cultivated plant with medicinal and nutritional benefits. This plant originated from the Northwest region of India where it was first discovered as a medicinal plant (Gowrishankar et al., 2010; Kamaraj & Rahuman, 2010). It belongs to the family of Moringaceae and has twelve other species (Ratshilivha et al., 2014). MO is known by several names in various regions where it is used, it is also referred to as drumstick tree or horseradish tree (Gopalakrishnan et al., 2016).

This plant has a wide range of uses and is grossly cultivated for its medicinal benefits in Asia and Africa (Yassa & Tohamy, 2014; Nayak et al., 2016). The leaf powder is used as a supplementary feeding program for the Integrated Child Development Scheme in India (Sixl & Drive, 2011). The leaves (Figure 10a), seeds (Figure 10b), bark, pod and flowers have high nutritional and phytochemical constituents (Chumark et al., 2008; Gupta et al., 2012; Vongsak et al., 2013).



Figure 10: *Moringa oleifera* leaves (a) and seeds with husk (b)

The leaves are also used to make soups (Nayak et al., 2016), as a result of the high protein content of the leaves it is added to animal feed as supplements (Fahey & Sc, 2005). MO is rich in simple sugars, glucosinolates and isothiocyanates (Fahey & Sc, 2005). The leaves are high in antioxidants such as zeatin, kaempferol, quercetin, isoquercetin, rutin, B- carotene, and ascorbic acid (Anwar et al., 2007; Sikder et al., 2013). These phytochemicals help to combat cellular damage when used. MO has the capability to manage and treat different ailments

considering its anti-inflammatory, antioxidant, antimicrobial, antifungal, anti-tuberculosis, analgesic, anti-diabetic,

antihypertensive, antibacterial and antifungal activities (Anwar et al., 2007; Brilhante et al., 2015; Georgewill et al., 2010; Kumar et al., 2010; Kunyanga et al., 2012; Verma et al., 2009). MO has been used in Ayurvedic medicine for centuries to prevent and treat various conditions such as diabetes, fever, gastric ulcer and bronchitis. Researchers have confirmed the presence of vitamins, minerals and protein in the leaves (Sixl & Drive, 2011). Our research aims at evaluating and assessing the active components and antioxidant capacity of MO in three different extraction solvents in order to ascertain the potentials of the active components in the management and treatment of diabetes.

3.2 Materials and methods

3.2.1 Chemicals

Methanol, petroleum ether and n-hexane, were purchased from Merck (Johannesburg, South Africa). Iron (III) chloride hexahydrate, 2,4,6-Tris(2-pyridyl)-s-triazine, hydrochloric acid, potassium peroxydisulfate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), chlorocresol green, chloroform, sodium carbonate, Folin-Ciocalteu's phenol, gallic acid, catechin hydrate, 4-(dimethylamino)-cinnamaldehyde (DMACA), quercetin and sodium dihydrogen monophosphate were procured from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

3.2.2 Ethics

This study was approved by the Research Ethics Committee (REC), Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology (CPUT) with approval reference number: CPUT/HW-REC 2014/AO8. All laboratory procedures were adhered to.

3.2.3 Plant Collection and Preparation

Green leaves of MO were collected from Forestry Research Institute of Nigeria (FRIN) botanical garden. The plant was authenticated by Mr A Adeyemo (a Botanist) and voucher specimen (number FHI-110287) was deposited in the Institute's herbarium. The leaves were washed, shade dried and crushed to powder. MO extracts were prepared from 1 kg of MO air-dried leaves which was first extracted with n-hexane for 24 hours then re-extracted with 80 % methanol (MET) and petroleum ether (PET) with continuous stirring for 48 hours. The solvents used for extraction were of analytical grade. The extracts were evaporated to dryness in a vacuum rotary evaporator under reduced pressure (Heidolph Instrument, Germany). For the aqueous extract (AQU) MO leaf powder was extracted with distilled water at temperature of 60–70 °C repeatedly, for 48 hours boiling, filtered with using Whatman no. 1 filter paper and then dried in a freeze dryer and stored at -4 °C.

3.3 Analysis

3.3.1 Determination of antioxidant capacity of various extracts of *Moringa oleifera*

For the determination of oxygen radical absorbance capacity (ORAC), the method of Ou et al. (2001) was utilised. Antioxidant capacity was performed on MO extract; Oxygen radical absorbance capacity (ORAC) was analysed using Ou's method. The reaction measured the capability of the extract to scavenge free radicals. A fluoroscan 96 well plate reader (Thermo Fisher Scientific, Waltham, MA, USA) was used to monitor reaction at 485 nm and 538 nm wavelengths for two hours. ORAC assay was carried out by addition of 138 µL working fluorescein, 12 µL of trolox used as the reference antioxidant, 12 µL of extracts, followed by 50 µL 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) which generates peroxy radical which initiated the onset of the reaction. Values were expressed as µmol trolox equivalent per gram dry weight (TE/g DW).

Ferric reducing antioxidant power (FRAP) was assayed using the protocol adapted from Benzie & Strain (1999). FRAP was assessed to determine the total antioxidant power of MO.

The addition of 10 μL of extract, 300 μL of FRAP reagent which included (10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), 300 mM acetate buffer at pH 3.6 (Saarchem, South Africa) in 0.1M HCl, 20 mM iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) (Sigma, Johannesburg, South Africa) and 6.6 mL distilled water gave a straw colour. L-Ascorbic acid was used as standard. All reagents were incubated for 30 minutes before reading was taken. Absorbance was measured at 593 nm. The results were expressed as μM ascorbic acid equivalent per gram dry weight (μM AAE/g DW).

Trolox equivalent antioxidant capacity (TEAC) was determined using the method described by Re et al. (1999). TEAC assay is used to measure the antioxidant capacity of MO, as compared to the standard, Trolox. A stock solution was prepared from 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS (8 mM) and potassium persulfate $\text{K}_2\text{S}_2\text{O}_8$ (3 mM) formed ABTS^+ which was prepared 24 h before the analysis was performed. Trolox was used as the standard, 25 μL of extract and 275 μL of ABTS mix was added to the well. Absorbance was read at 734 nm and results were expressed as μmol TE/L.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined using the method described by Sharma & Bhat, (2009). The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored in a dark room until use. Absorbance was read at 517 nm.

3.3.2 Total polyphenols, total alkaloids, flavanols and flavonols content of *Moringa oleifera* leaf extract

Total polyphenols, total alkaloids, flavanols and flavonols contents of MO extracts were determined. Total polyphenols content was determined using Folin-Ciocalteu protocol according to the procedure of Singleton and colleagues (1999). Folin-Ciocalteu reagent and sodium carbonate were mixed together and allowed to rest at room temperature for 24 hrs. The solution was added to the extracts with gallic acid as standard and read at an absorbance of 765 nm. Results were expressed as mg GAE/g dry weight.

Total alkaloids (Fadhil et al., 2007) and flavanols (McMurrough & McDowell, 1978) contents were determined in MO extract by colorimetric methods. Total flavonol content was extracted with 2 % HCl, 95 % ethanol and quercetin as standard (Mazza et al., 1999). Results were measured spectrophotometrically at 360 nm and expressed as mg QE/L. All absorbances were read with the Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.3.3 High-Performance Liquid Chromatography (HPLC) Analysis of *Moringa oleifera* methanol extract

Phenolic and flavonoid content of methanol extract of MO was determined by a high-performance liquid chromatography-HPLC (Agilent Technology 1200 series, Bellefonte, USA) using a diode array detector and a C18 column (5 μ m (4.6 mm x 150mm i.d.)). Twenty (20) μ l of sample was automatically injected into the column and isocratic elution performed on a mobile phase consisting of methanol: acetone (9:1, v/v) with the flow rate set at 1 mL/min. Detection was performed at wavelengths of 280, 320 and 360 nm. Peaks were identified based on the retention time of specific phenolic and flavonoid standards (Vongsak et al., 2012). Concentrations were calculated according to the equation: area of standard/area of sample X 20 μ g/ml. Results were expressed as mg/ml.

3.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prisms 7.0 (GraphPad for Windows, La Jolla, CA, USA). Comparison among group means was analyzed with one-way analysis of variance (ANOVA) and Turkey multiple-comparison as the post hoc test. Values were expressed as mean \pm standard deviation, and a value of $p < 0.05$ was considered statistically significant.

3.5 Results

3.5.1 Total antioxidant capacity and phytochemical content of various extract of *Moringa oleifera*

The antioxidant capacity and phytochemical content of methanol (MET) and petroleum leaf extracts (PET) of MO are shown in Table 2. The antioxidant capacity was evaluated using ORAC, FRAP, TEAC and DPPH assays. Antioxidant capacity of MO was highest in the aqueous extract, followed by methanol extract and then petroleum ether extract. Results from the antioxidant capacity of the aqueous extract are shown in Table 2 below.

The phytochemical content of MO extracts was assessed for total alkaloids, total polyphenols, flavanols and flavonols content. The phytochemical content of MO evaluated revealed the presence of total alkaloids with the highest concentration in petroleum ether extract (8.77 ± 0.83 mg CE/L) while total polyphenols (5481.02 ± 91.32 mgGAE/L) and flavonols (373.70 ± 5.71 mg QE/L) contents were the highest in the AQU extract. Flavanol was absent in all three extracts.

Table 2: Total antioxidant capacity and the phytochemical content of various extract of *Moringa oleifera*

<i>Moringa oleifera</i> extracts			
Constituents	Aqueous Extract	Methanol extract	Petroleum ether extract
ORAC	4941.02 \pm 55.55	3652.10 \pm 113.32	30.5.80 \pm 35.53
μmol TE/L			
FRAP	4450.01 \pm 89.31	1736.03 \pm 3.08	65.06 \pm 5.13
μmol AAE/L			
TEAC	2167.18 \pm 7.15	1697.50 \pm 34.21	224.10 \pm 30.63
μmol TE/L			
DPPH	1433.20 \pm 11.02	850.80 \pm 6.04	82.24 \pm 12.08
μmol TE/L			
Total alkaloids	2.51 \pm 0.13	3.58 \pm 0.08	8.77 \pm 0.83
mg CE/L			
Total polyphenols	5481.02 \pm 91.32	2328.00 \pm 165.70	72.97 \pm 26.06
mgGAE/L			
Flavanols	NA	NA	NA
mg QE/L			
Flavonols	373.70 \pm 5.71	155.30 \pm 6.49	193.40 \pm 5.71
mg QE/L			

Values are presented as Mean \pm SD. TE (trolox equivalent), AAE (ascorbic acid equivalent), CE (catechin equivalent), GAE (gallic acid equivalent) and QE (quercetin equivalent).

3.5.2 HPLC determination of specific flavonoid content in *Moringa oleifera* methanol leaf extract at 370 nm

HPLC determination of specific flavonoid content in MO methanol leaf extract at 370 nm is shown in Table 3. Results from the HPLC analysis identified specific flavonoids in MO methanol leaf extract. Quantitative studies revealed the presence of quercetin (17.03 µg/ml), rutin (9.55 µg/ml) and myricetin (108.02 µg/ml) at a wavelength of 370 nm. The HPLC chromatogram (Figure 11) of MO extract shows the presence of peaks consistent with reference standards. Figure 12 shows the chemical structure of quercetin, rutin and myricetin in MO extract identified at 370 nm.

Table 3: Flavonoid content in *Moringa oleifera* methanol extract at 370 nm

	Wavelength λ	Concentration (µg/ml)	Retention time (RT) minutes	Area under curve (AUC)
Quercetin	370	17.03	21.66	979
Rutin	370	9.55	17.89	483.2
Myricetin	370	108.02	19.29	858

HPLC analysis of methanol extract of *Moringa oleifera* showing wavelength, concentration, retention time, and area under the curve. All values are in 20 mg/ml plant material.

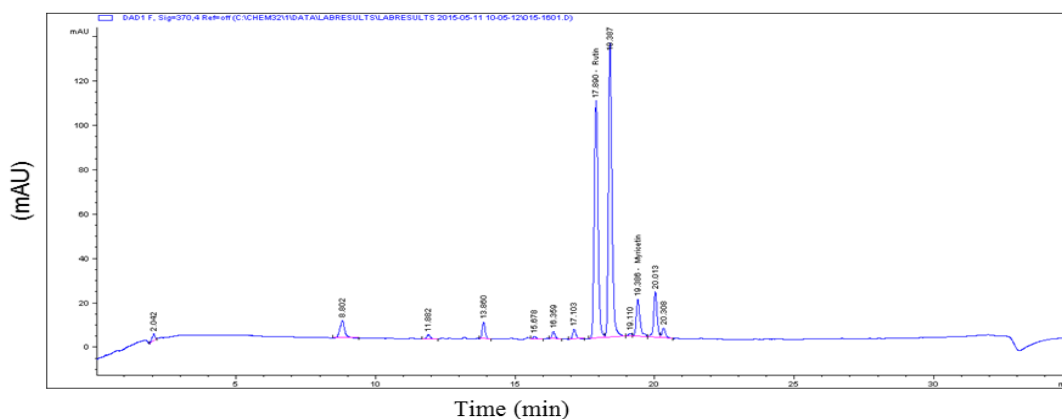


Figure 11: HPLC chromatogram of flavonoids identified in *Moringa oleifera* methanol extract analysed at a wavelength of 370 nm.

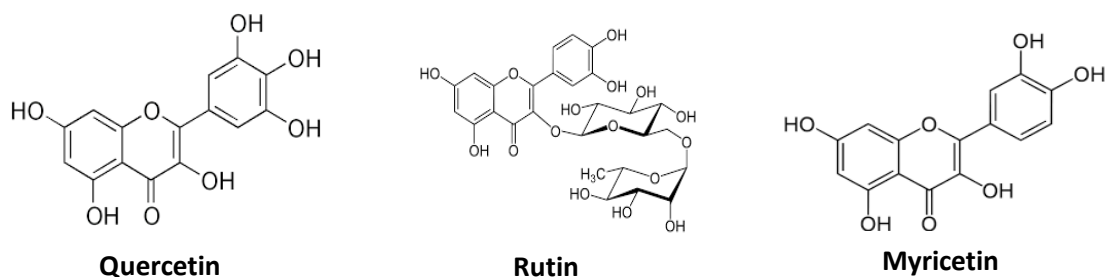


Figure 12: Chemical structure of quercetin, rutin and myricetin in *Moringa oleifera* extract identified at 370 nm

3.5.3 HPLC determination of specific phenolic content of *Moringa oleifera* methanol extract at 320 nm

Table 4 shows the identification of specific phenolic content of MO extract at 320 nm. Quantitative studies indicated the presence of phenolic acid such as chlorogenic acid (250 µg/ml), caffeic acid (8,119 µg/ml) and coumaric acid (15,740 µg/ml) at a wavelength of 320 nm. The HPLC chromatogram (Figure 13) of MO extract revealed the presence of peaks consistent with reference standards. Figure 14 shows the chemical structures of phenolics acids chlorogenic acid, caffeic acid and coumaric acid identified in MO extract analyzed at a wavelength of 320 nm.

Table 4: Phenolic acid content in *Moringa oleifera* methanol extract at 320 nm

	Wavelength λ	Concentration (µg/ml)	Retention time (RT) minutes	Area under curve (AUC)
Chlorogenic acid	320	250	11.41	719.5
Caffeic acid	320	8,119	12.64	1705
Coumaric acid	320	15,740	12.12	2361

HPLC analysis of methanol fraction of *Moringa oleifera* showing wavelength, concentration, retention time, and area under the curve. All values are in 20 mg/ml plant material.

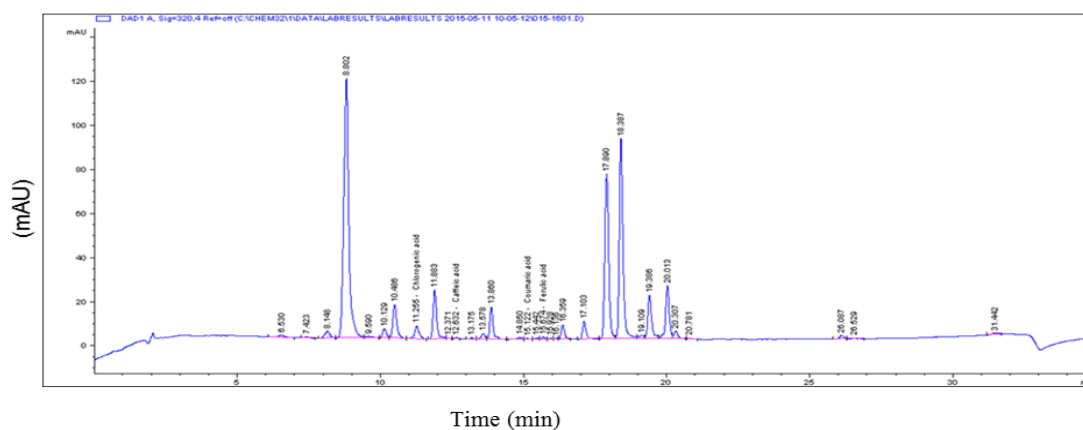


Figure 13: HPLC chromatogram of phenolic acid identified in *Moringa oleifera* methanol extract analysed at a wavelength of 320 nm

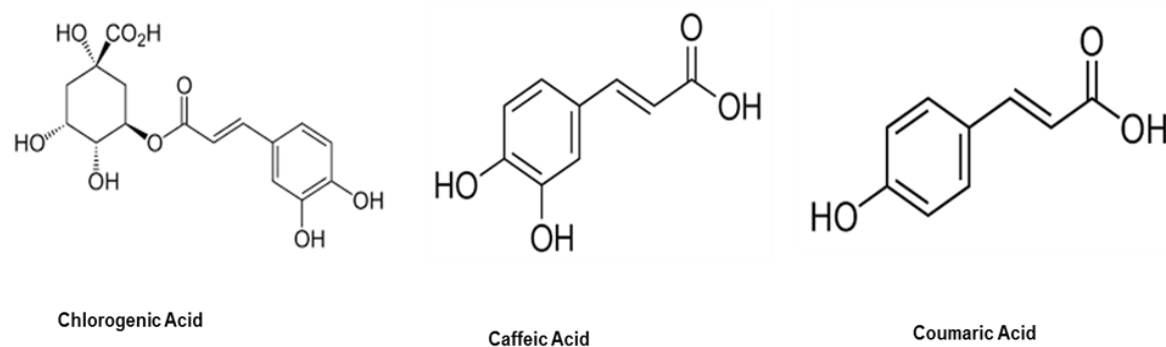


Figure 14: Chemical structures of phenolics acids, chlorogenic acid, caffeic acid and coumaric acid identified in *Moringa oleifera* extract analysed at a wavelength of 320 nm.

3.5.4 HPLC determination of other phenolic contents in *Moringa oleifera* methanol leaf extract

Determination of other phenolic contents in MO methanol leaf extract shown in Table 5: The HPLC chromatogram of MO extract revealed the presence of peaks consistent with reference standards. Our studies indicated the presence of catechin, cinnamic acid, gallic acid, vanillin, sinapic acid, ferulic acid and luteolin as shown Figure 15 at 280 nm. HPLC chromatogram at 320 nm, 370 nm and above revealed the presence of peaks consistent with reference standards. Chromatogram (Figure 15) identified the presence of peaks consistent with reference standards.

Table 5: Other phenolics in *Moringa oleifera* methanol extract identified at 280 nm, 320 nm and 370 nm

	Wavelength λ	Retention time (RT) minutes	Area under curve (AUC)
Catechin	280	12.79	265
Cinnamic acid	280	20.62	2926
Gallic acid	280	5.41	472.3
Vanillin	280	13.25	1547
Sinapic acid	320	15.64	1673
Ferulic acid	320	15.65	1837.1
Luteolin	370	22.34	725

HPLC analysis of methanol fraction of *Moringa oleifera* showing wavelength, retention time, and area under the curve. All values are in 20 mg/ml plant material

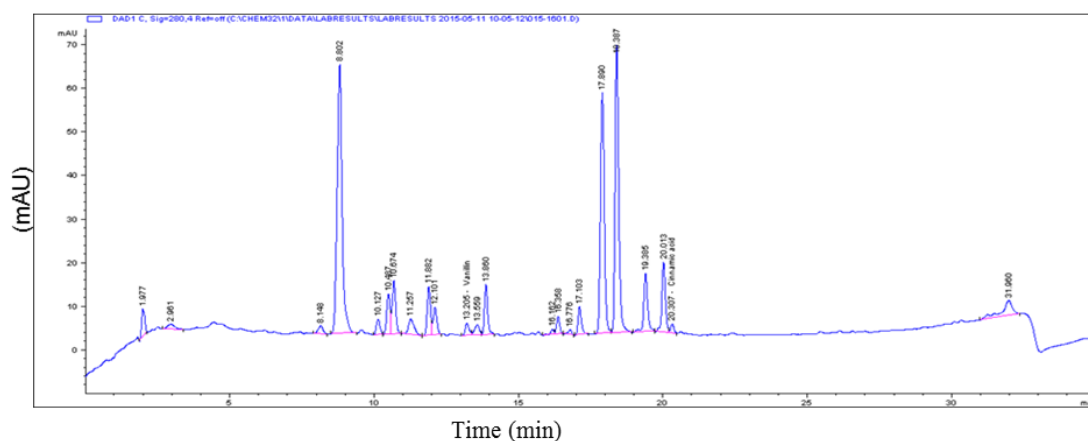


Figure 15: HPLC chromatogram of phenolic content identified in methanol leaf extract of *Moringa oleifera* analysed at a wavelength of 280 nm.

3.6 Discussion

Crude aqueous extracts, petroleum ether extract and methanol extract of MO-derived from the leaf powder were investigated for their antioxidant capacity and phytochemical content. Antioxidants and phytochemicals present in MO were quantified, identifying this plant as a rich source of polyphenols.

The antioxidant capacity of AQU, MET and PET of MO was evaluated using ORAC which measures the ability to scavenge oxygen radicals, and TEAC based on the inhibition by antioxidants of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical (ABTS[•]) by converting it into a colourless product from the initial bluish-green colour (Re et al., 1999). The FRAP assay measures antioxidant capacity in samples through the reduction of ferric iron (Fe³⁺) to ferrous iron (Fe²⁺) via reduction reaction to form ferrous-tripyridyltriazine thus forming a coloured complex, measured at 593 nm. DPPH is used to evaluate the antioxidant capability to scavenge free radicals.

DPPH radical scavenging activity results showed antioxidant capacity of MO which was highest in AQU extract, followed by MET extract and PET extract. This implies that MO extract contains bioactive compounds able to donate hydrogen to free radicals thereby removing odd electron that are responsible for free radical generation. Aqueous extract showed high

antioxidant capacity as compared to other extracts. MO offers protection against oxidative damage caused by free radicals, thus acting as a free radical scavenger (Halliwell, 2011). It further proffers a protective effect to cells, minimising the destructive effect of free radicals.

The phytochemical content of MO evaluated revealed the presence of alkaloids which was the highest in PET extract, while total polyphenol and flavonol contents were highest in AQU extract. PET was identified as the best extraction solvent for alkaloids in MO, this may be due to the fact that alkaloids dissolve poorly in water and dissolve well in PET which is a non-polar lipophilic solvent and AQU extracts concentration was high in total polyphenols and flavonols owing to the polarity of phenol and flavonols determined. Phytochemical screening of the MO extract using the three solvents confirmed the presence of polyphenols, alkaloids and flavonols content and absence of flavanols. The presence of phytochemicals in MO plant extract shows antimicrobial, anthelmintic, and antidiarrhoeal activities.

The potency of MO may be due to the various phytochemicals such as total polyphenols, total alkaloids and flavonols and antioxidants identified in the leaves of this plant. This explains why MO is used to treat various diseases such as malaria, typhoid, inflammatory-related ailments, ulcers, arthritis, piles, erectile dysfunction, diabetes and associated diabetic complications (Verma et al., 2009; Kumar et al., 2010). The antioxidant and phytochemical contents of MO extract are high which may be responsible for its medicinal activities that offer beneficial effect as a good therapeutic agent to combat numerous diseases.

The HPLC chromatogram of MO methanol extract revealed the presence of flavonoids; quercetin, rutin and myricetin at a wavelength of 370 nm. Quercetin is a potent antioxidant and protects beta-cells from STZ-induced oxidative damage (Sikder et al., 2013). Rutin is a glucoside of quercetin called quercetin-3-O-rutinoside. It is an effective antioxidant. Myricetin is the most abundant flavonoid identified in MO leaf extract. Quercetin, rutin and myricetin are sources of plant antioxidants and health promoting compounds and have all been

demonstrated to proffer some protective effect against cardiovascular disease, stress and diabetes, which may be responsible for its antidiabetic properties (Verma et al., 2009).

Additional constituents identified include phenolics such as chlorogenic acid, caffeic acid and coumaric Acid at a wavelength of 320 nm. Other constituents such as catechin, cinnamic acid, ferulic acid, gallic acid, luteolin, sinapic and vanillin were also identified in MO leaf extract. Chlorogenic acid present in MO is an ester of quinic acid and caffeic acid, it is essential in glucose metabolism (Heleno et al., 2015). Disease prevention ability of MO is attributed to its phytochemical content elucidated in this study.

MO is possibly one of the most useful traditional medicinal plants in Africa. In recent times, attention has been drawn to this plant as a result of its phytochemical and antioxidant contents/properties elucidated in our study. MO exerts its ameliorative effect by reducing oxidative stress, endothelial cell dysfunction, kidney lipid peroxide and increasing antioxidant capability. This effect may be related to MO's phytochemical composition which appears to commence a recovery process from associated cell related damage. Although several studies have been undertaken, no study has compared the antioxidant and phytochemical properties of aqueous, methanol and petroleum ether extract of MO. Quantitative analysis of the various solvents of this extract revealed the aqueous extract as the best extraction solvent for antioxidants followed by methanol. Furthermore, the study revealed that petroleum ether was the best method for the extraction of alkaloids from MO.

3.7 Conclusion

MO possesses high phenolic content (antioxidant) activities as confirmed in this study. This accounts for its medicinal activities that offer beneficial effects in scavenging of ROS which is usually high in untreated chronic disease such as DM.

Competing interests

The authors declare no conflict of interest regarding the publication of this manuscript.

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CHAPTER FOUR

4 Assessment of the anti-hyperglycaemic, anti-inflammatory and antioxidant activities of the methanol extract of *Moringa oleifera* in diabetes-induced nephrotoxic male Wistar rats

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ABSTRACT

Background Diabetes mellitus (DM) is an endocrine disease of impaired insulin secretion or insulin resistance. A deficiency in insulin secretion or action results in hyperglycemia with metabolic disorders. *Moringa oleifera* (MO) is available in the tropics with a variety of ethnomedicinal importance. The leaf of this plant has been reported to possess antioxidant and medicinal properties which may be helpful in the treatment and management of diabetes and complications linked to diabetes.

Methods: Diabetes was induced in rats by a single intraperitoneal dose of streptozotocin` (55 mg/kg) and animals were treated with methanol extract of *Moringa oleifera* (250 mg/kg b.wt) for 6 weeks. Forty-eight (48) adult male Wistar rats were randomly divided into four (4) groups: Normal control (NC), *Moringa oleifera* treated control rats (NC+MO), Diabetic rats (DM) and *Moringa oleifera* treated diabetic rats (DM+MO). Estimation of antioxidant capacity, total polyphenols, flavonoids and flavonols content of *Moringa oleifera* extracts were performed and serum biochemical markers were evaluated, antioxidants enzymes: catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) activities, glutathione (GSH) concentration and inflammatory biomarkers were determined in the kidney homogenate of these rats. Kidney histology was carried out.

Results: Induction of diabetes via STZ, generated increased reactive oxygen species (ROS) and hyperglycaemia evident by the significant increase in glucose levels, lipid peroxidation (MDA) and inflammatory biomarkers. Results showed high antioxidant capacities of MO extract, improved serum biochemical markers whilst lipid peroxidation (MDA) levels reduced in the kidney of non-diabetic and diabetic rats after MO treatment when compared to diabetic control. Subsequent administration of MO led to an increased concentration of serum albumin, globulin and total protein and improvements in CAT, SOD, GSH, GPx, TNF- α and IL-6. Kidney sections of diabetic rats revealed severe renal damage showing interstitial nephritis at the cortical area of the kidney. However, administration of MO showed appreciable improvements to these alterations revealing mild vascular congestion of the glomerulus.

Conclusion: MO contains potent phytochemical constituents that offer protective effects against diabetic-induced toxicity, improves antioxidant status, reduced pro-inflammatory cytokines and may, therefore play a significant role in the treatment and management of diabetes particularly in developing countries such as in Africa where the majority of people cannot afford orthodox medicine.

Keywords: *Moringa oleifera*, diabetes, methanol extract, streptozotocin, hyperglycemia, nephrotoxicity, phytochemical, antioxidant, flavonoids, inflammatory.

4.1 Introduction

The increasing prevalence of diabetes in both developed and developing countries has challenged scientists to further conduct research in sourcing for potent therapeutic agents from natural sources for more efficient usage in the treatment and management of diabetes [1]. There is an emerging global epidemic of diabetes that can be traced back to a rapid increase in weight, obesity and sedentary lifestyle [2]. Diabetes is a degenerative disease of the blood glucose system, characterized by pancreatic beta cells deficiency to produce insulin or sufficient insulin resulting in chronic hyperglycemia, which is associated with long-term microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (cardiovascular) complications [2]. Hyperglycemia caused by oxidative stress is implicated in the onset and progression of diabetes and if left untreated, can lead to severe complications [3]. Diabetes is predicted to become the seventh leading cause of death in the world by 2030 and total death from diabetes are projected to rise by more than 50% in the next 10 years [4].

Individuals with diabetes have high levels of inflammatory cytokines, activation of leukocytes and increased tissue fibrosis [5]. Accumulation of ROS leads to oxidative stress which is associated with increased damage to β -cells and biomolecules [6]. Reactive oxygen species are highly reactive atoms with unpaired electrons in their outer orbital such as superoxide anion, singlet oxygen, hydroxyl radical, peroxy radical and carboxyl radical [7,8]. ROS are produced by aerobic metabolism, electron transport activity (releases unpaired electrons), a by-product of the normal enzymatic reaction, as well as during inflammatory response, stress, and human activities including pollution, alcohol consumption and drugs [9].

Potential damage to biological molecules, cell membranes, cellular lipids, inflammation, β -cells destruction, and eventually cell death are mediated by ROS through direct reaction [10, 11]. Unfortunately, over-production of ROS has been implicated leading to extremely harmful effects such as nephropathy, neuropathy, ketoacidosis, retinopathy, cancer, arthritis, coronary heart disease, peroxidation of lipids in the bloodstream, heart diseases, early ageing, damage

to DNA and proteins (leading to errors in replication) [12-14]. Furthermore, over-accumulation of ROS disrupts the antioxidant mechanism giving rise to a cascade of deleterious events, thereby inactivating antioxidant enzymes and stimulating glycation of proteins resulting in diabetic complications [15, 16].

Given that increased levels of free radicals such as reactive oxygen species (ROS), inflammatory mediators and apoptotic proteins are associated with DM, scavenging activities of MO can help to modulate inflammatory molecules in diabetic rats protecting tissues from oxidative stress and preventing the development of diabetic complications [17,18]. Previous studies have shown that aqueous, methanol and ethanol extracts of MO leaves possess a wide range of biological activities such as antioxidant, antiulcer, analgesic, radioprotective, antihypertensive, and immunomodulatory actions in-vivo [19,20]. Researchers have reported hypoglycemic and anti-inflammatory effects of MO in normal and diabetic male rats and showed the ability of MO to lower blood glucose level and reduce free radical activity [21-24].

Lipid peroxidation results from free radical chain reaction causing deterioration in the lipid of the cell membrane; affecting the structural and physiological integrity of cells. This is seen in diabetic conditions, kidney diseases and disruption in blood vessels [25,26]. Antioxidants (non-enzymatic and enzymatic) help to mop up free radicals and ROS, preventing lipid peroxidation thereby reducing deleterious effects caused by these species [27-29].

Moringa oleifera (MO) has rich antioxidant content and diverse therapeutic abilities. The previous investigation identified MO with the ability to prevent the occurrence and complications of diabetic-induced kidney injury through its protective effects on the oxidative status and inflammatory cytokines in the kidney of diabetic rats [30]. This plant has been reported to have some analgesic, anti-diabetic, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antibacterial properties and plays beneficial roles in modern medicine [31-33].

Remarkably, studies with experimental animals *in vivo* on markers of oxidative stress and correlation with antioxidant properties *in vitro* systems have been reported though not extensively. *In vivo* studies revealed the antioxidant capacity of the aqueous extract of MO leaf to possess the potency of increasing the antioxidant status and reduce lipid peroxidation in a dose-dependent manner while *in vitro* demonstrated high antioxidant capacity thereby showing protective effects against ROS [20,34]. Edoga and others [35] reported anti-diabetic and hypotensive activities of MO in albino rats, as the plant played a role as a hypoglycemic agent in lowering blood glucose levels and preventing further cellular damage.

Previous studies on the extracts of MO using chromatographic and spectroscopic techniques revealed the presence of notable phenolic compounds such as kaempferol, quercetin, catechin, gallic acid, caffeic acid, p-coumaric acid, vanillin, ferulic acid, protocatechuic acid, cinnamic acid and epicatechin [17,36]. These secondary metabolites identified from MO extract have been linked to various biological profiles including antioxidant, antituberculosis, analgesic, anti-cancer, anti-diabetic, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antibacterial and antimicrobial and antimalarial activities exhibited by this plant [36].

Some histological examination of the pancreatic section of diabetic rats revealed degenerative changes in β -cells which were significantly reversed after treatment with extract MO [1,37]. Anti-hepatotoxic effects of MO and *Vernonia amygdalina* (VA) extracts in streptozotocin (STZ) induced diabetic rats revealed that single and combined extracts of MO and VA have hepatoprotective effects and may be effective in reducing liver damage [38].

This study was planned to examine the anti-hyperglycaemic, anti-inflammatory and anti-oxidative effects of MO in male Wistar rats. This study links diabetes with increased lipid peroxidation and oxidative damage. Antioxidant capacity status of MO has not been extensively estimated, and specific phytochemicals in MO methanol leaf extract have not been comprehensively evaluated. These were explored in the study with the overarching aim of

clarifying the potential of MO as a potential source of antioxidant and bringing to fore its stunning capabilities that could feasibly revolutionise pharmacological products in the treatment and management of diabetes.

Furthermore, due to MO's numerous scientific and health claims, it is appropriate to further investigate the potential treatment regime of MO on STZ-induced diabetes in animal model which can be used to advance clinical techniques on its ameliorative activities. It is anticipated that the outcome will support the ethnomedicinal information of this plant, especially among rural communities.

4.2 Methods

4.2.1 Chemicals

Streptozotocin (STZ), quercetin, 6-hydroxydopamine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2-thiobarbituric acid (TBA) and β -nicotinamide adenine dinucleotide phosphate reduced tetra-sodium salt [NADPH] were obtained from Sigma chemical company (Johannesburg, South Africa). Methanol, petroleum ether, malondialdehyde bis (diethyl acetal) (MDA) and hexane, were purchased from Merck (Johannesburg, South Africa). Other chemicals were of the highest grade and procured from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

4.2.2 Collection of plant material

Fresh MO leaves and flower were obtained from botanical garden of Forestry Research Institute of Nigeria (FRIN) in October 2014. The plant was authenticated by a plant taxonomist Mr. A.A Adeyemo with a voucher specimen (FHI-110287) deposited in the Institute's herbarium.

4.2.3 Extract Preparation

Green leaves of MO were washed, air dried and blended to powdery form. Extracts were prepared from 1 kg of MO powder via continuous stirring in n-hexane for 24 h. The residue was re-extracted in 80 % (v/v) methanol (Merck, Johannesburg, South Africa) at room temperature for 24 h. The methanol extracts were evaporated to dryness *in vacuo* using rotary evaporator (Heidolph Instrument, Germany) and stored at -4 °C for use and phytochemical screening.

4.2.4 Ethical statement

All experimental protocols described in this study was approved by the Faculty of Health and Wellness Sciences, Research Ethics Committee (REC) of Cape Peninsula University of Technology with REC approval reference number: CPUT/HW-REC 2014/AO8. All animals received humane care according to the principles of laboratory animal care of the National Institutes of Health Guide for the care and use of laboratory animals of the National Academy of Science (NAS) publication no. 80-23, revised 1978.

4.3 Study design

4.3.1 Experimental animals

Experimental animals used for this study were adult male Wistar rats weighing about 200 g and 250 g and aged 10 weeks. The animals were housed at Stellenbosch University Animal Research Facility, Tygerberg, South Africa and used for the experiment. Forty-eight (48) rats were randomly divided into four (4) groups. Rats were kept in a well-ventilated animal facility in stainless steel cages (beddings composed of ground sterilized maize cobs) with 5 rats per cage to allow free mobility. Rats were fed with standard rat chow (Aquanutro, Malmesbury, South Africa) and water *ad libitum*. A conducive temperature of 22 ± 2 °C, humidity 55 ± 5 % and normal period (12 h light / 12 h dark) was maintained.

4.3.2 Induction of diabetes

Diabetes was induced in rats by a single intraperitoneal (IP) injection of freshly prepared streptozotocin (STZ, Sigma, St. Louis, USA) in citrate buffer (0.1 M pH 4.5) to overnight fasted rats at a dose of 55 mg/kg [6]. Blood was obtained from the rat's tail to confirm a diabetic state using a glucometer (Accu-Check, Roche, Germany) and test strip. A stable fasting blood glucose level of >18 mmol/L three days post-STZ injection confirmed hyperglycemia, and only diabetic rats were included in the study [6]. MO administration commenced on the fourth day and this was considered as the first day of treatment.

4.3.3 Treatment

Based on preliminary investigations, a dose of 250 mg/kg MO extract was the most suitable, and this is consistent with the dosage administered by Kar et al. (2003), Efiang et al. (2013) and Tabassum et al. (2013). The forty-eight rats were randomly divided into four groups of twelve rats each; NC-Normal non-treated control, MO-*Moringa oleifera* treated control rats, DM- diabetic rats and DM + MO- *Moringa oleifera* treated diabetic rats. NC and DM (control groups) received distilled water while MO and DM+MO (experimental groups) received *Moringa oleifera* extract at a dose of 250 mg/kg/b.wt via oral gavage daily for six (6) weeks.

At the end of the treatment, rats were fasted overnight and anaesthetized with sodium pentobarbital intraperitoneal injection (60 mg/kg). Sodium pentobarbital was used to ensure the unconsciousness of rats while death occurred while guaranteeing rapid and painless death. This procedure was carried out in the animal house. Blood samples were obtained via the rat's abdominal aorta into a lithium heparin plasma separator tubes and serum clot activator tubes. The whole kidneys were quickly excised from each rat, washed in ice-cold phosphate-buffered saline, blotted, weighed and frozen in liquid nitrogen.

4.3.4 Blood and kidney homogenate preparation

Blood samples were centrifuged at 4000 g for 10 minutes 4 °C to obtain plasma and serum and stored at -80 °C. Kidneys (200 mg) were homogenized on ice in 2000 µL ice-cold

phosphate buffer saline (PBS, 50 mM pH 7.5). Homogenates were centrifuged at 15000 rpm for 10 minutes at 4 °C. The supernatants were aliquoted and stored at -80 °C for estimation of biochemical parameters.

4.4 Experimental Analysis

4.4.1 *Relative kidney weight*

The relative kidney weight was estimated by comparing the kidney weight to the body weight of the same rat.

$$\text{Relative Kidney weight (mg/100 g body weight)} = \frac{\text{Kidney weight (g)}}{\text{Total body weight (g)}} \times 100 \%$$

4.4.2 *Plasma glucose determination*

Plasma collected from blood was used for determination of plasma glucose level using a Randox kit from Randox Laboratories Limited (Crumlin, United Kingdom). A standard protocol was followed according to the manufacturers operating procedures.

4.4.3 *Antioxidant capacity of Moringa oleifera methanol extracts*

Oxygen radical absorbance capacity (ORAC)

Antioxidant capacity was performed on MO methanol extract; Oxygen radical absorbance capacity (ORAC) of MO extracts was analysed according to Ou et al. [39]. Values were expressed as μmol trolox equivalent per gram dry weight (TE/g DW).

Ferric reducing antioxidant power (FRAP)

FRAP was assessed to determine the total antioxidant power of MO using the protocol adapted from Benzie & Strain [40]. The results were expressed as μM ascorbic acid equivalent per g dry weight (μM AAE/g DW).

Trolox equivalence antioxidant capacity (TEAC)

TEAC measures the antioxidant capacity of MO, as compared to the standard, Trolox. TEAC assay was carried out using the method described by Re et al. [41]. Results were expressed as μmol TE/L and absorbance read at 734 nm.

4.4.4 Phytochemical investigation: Total polyphenol, flavonoids and flavonol content of *Moringa oleifera* methanol extract

Total polyphenols content was determined in extracts of MO using Folin–Ciocalteu protocol; folin's reagent; Sodium carbonate and extract were added to wells with gallic acid as standard. Results were expressed as mg GAE/g dry weight and absorbance read at 765 nm [42].

Total flavonoid content was determined in MO extract by a colourimetric method described by Singleton [42]. Results were expressed as quercetin standard equivalent mg QE per gram extract and absorbance read at 510 nm. Total flavonol content was extracted with 2 % HCl, 95 % ethanol and quercetin as standard. Results were measured spectrophotometrically at 360 nm and expressed as mg/QE/g dry weight [43]. A Multiskan 96 well plate reader (Thermo Electron Corporation, Beverly, MA, USA) was used to read all samples.

4.4.5 Serum total protein, albumin and globulin determination

Total protein concentration was estimated in the serum with kit using an automated Randox Daytona from Randox Laboratories Limited (United Kingdom). A coloured complex was formed through a reaction that occurs between protein and cupric ions in alkaline medium. All protocols were in accordance with the manufacturer's instruction.

Serum albumin level was determined using kits from Randox Laboratories Limited (United Kingdom). The principle is based on its ability to bind to 3, 3', 5, 5'-tetrabromo-m cresol sulphonphthalein. The complex formed was measured at 578 nm.

Globulin level was determined using the formula:

$$\text{Globulin} = \text{Total protein} - \text{Albumin}$$

4.4.6 Lipid peroxidation and activities of antioxidant enzymes in the kidney

Lipid peroxidation was determined in the kidney homogenate by measuring the conjugated dienes and malondialdehyde using high-performance liquid chromatography-HPLC. (Agilent 1200 Technologies, Bellefonte, USA) with a diode array detector, 5 µm column C18 (4.6 mm

x 150 mm). 50/50 methanol: water was used as the mobile phase, run rate at 10 mins and 1ml/min flow rate.

Fluorometric detection of Malondialdehyde (MDA) was performed with excitation at 532 nm and emission at 552 nm. MDA, an end product of lipid peroxidation was determined using Khoschsorur's method [44]. MDA-TBA adduct peaks were calibrated with the MDA standard. Malondialdehyde was used as the standard and results expressed as $\mu\text{mol MDA/g}$ for kidney homogenates.

Superoxide Dismutase (SOD): Activity of SOD was assessed using a 96 well Multiskan plate Thermo Electron Corporation reader (Beverly, MA, USA) modified for a microplate reader at 490 nm and expressed as the amount of protein [mg] required to produce a 50 % inhibition of auto-oxidation of 6-hydroxydopamine [45].

Catalase (CAT): Activity of CAT was determined spectrophotometrically at 240 nm. This was done by monitoring the decomposition of H_2O_2 and expressed as $\mu\text{mole H}_2\text{O}_2/\mu\text{g protein}$ [46].

Total glutathione (GSH): The level of total glutathione GSH in the kidney was estimated by the method described by Asensi et al. [47]. All the analyses were done using a 96 well plate and read with the Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA).

Glutathione Peroxidase (GPx): Level of GPx activity in the kidney was determined spectrophotometrically at 340 nm and expressed as $\mu\text{moles NADPH/ mg protein}$ [48].

4.4.6.1 Histopathology

Histopathology of the kidney tissues was performed at Stellenbosch University, Department of Anatomy and Histology (Tygerberg, Cape Town, South Africa). Kidney tissues were fixed in 10% buffered formalin, dehydrated in ethanol and embedded in paraffin using the Leica EG1160 embedder (Buffalo Grove, IL, United States). Tissue blocks were sectioned to 5 μm and stained with hematoxylin and eosin for histological examination using a Leica RM2125

RTS microtome (Leica Microsystems, Inc., Buffalo Grove, IL, United States). The slides were examined by a pathologist under a light microscope at a magnification of X400.

4.4.7 Determination of Tumour necrotic factor (TNF- α) and Interleukin (IL-6) in the kidney

The concentration of inflammatory biomarkers; tumour necrotic factor (TNF- α) and interleukin (IL-6) in the kidney were determined using Millipore's MILLIPLEX[®] MAP rat cytokine magnetic bead panel kit (Millipore Cooperation, MA, USA). The protocol was followed according to the manufacturer's instruction. The assay was performed on the Bio-plex[®] platform (Bio-Rad) and Bio-Plex Manager TM software, version 6.0 was used for analysis.

4.5 Statistical analysis

The values were analyzed using GraphPad Prisms 5.0 (GraphPad software for Windows, San Diego, CA) to calculate the mean \pm SD (standard deviation). One way analysis of variance (ANOVA) was used to compare differences in multiple groups with Newman-Keuls Multiple Comparison used as a post hoc test, and $p < 0.05$ was considered statistically significant between control and test means.

4.6 Results and discussion

The rapid discovery of various medicinal plants and natural products with anti-diabetic potentials has provided a remarkable intervention in the history of many diseases including diabetes [49]. The basis for the use of a number of plants as novel remedies for diabetic complications cannot be overemphasized [50,51].

Hyperglycemia caused by oxidative stress has been shown to be directly involved in the onset and progression of diabetes, leading to various complications such as cardiovascular diseases, nephropathy, amputation of limbs and blindness [52-54]. The mechanism of STZ (C₈H₁₅N₃O₇) as a toxicant used to induce hyperglycemia in experimental animal involves its

toxic effects on the beta cells of the pancreatic islet [55]. Consequently, ROS are formed during this process and a cascade of reactions occur leading to increased levels of superoxide radicals, hydrogen peroxide, and hydroxyl radicals with potentially damaging effects on cell macromolecules in the animals [29,56].

4.6.1 **Effect of *Moringa oleifera* on kidney weight, relative kidney weight and plasma glucose level of rats exposed to STZ-induced diabetes**

The effect of MO on kidney weight, relative kidney weight and blood glucose levels of rats is shown in Table 6. The difference in kidney weight between non-diabetic treated rats (NC+MO) and normal control is not significant. However, *Moringa oleifera*-treated control rats (NC+MO) showed a significant ($p < 0.05$) decrease when compared to the diabetic control (DM). Kidney weights of diabetic control rats increased significantly ($p < 0.05$) when compared to normal control (NC). After treatment of diabetic rats (DM+MO) with MO, a significant ($p < 0.05$) decrease was observed when compared to diabetic control (DM). Similar results were observed in the relative kidney weight. Diabetes induction markedly elevated blood glucose levels. MO treatment of diabetic rats significantly lowered plasma glucose levels, although treatment did not normalize blood glucose levels. Similarly, MO treatment lowered blood glucose levels of normoglycemic rats in comparison to normal control.

Table 6: Effect of *Moringa oleifera* on kidney weight, relative kidney weight and plasma glucose level

	NC	MO	DM	DM+MO
Kidney weight (g)	1.90±0.17	1.80±0.17 ^b	2.38±0.18 ^a	2.13±0.20 ^{ab}
Relative kidney weight (g/100 g)	0.60±0.04	0.57±0.03 ^b	1.10±0.08 ^a	1.03±0.08 ^{ab}
Body weight (g)	313.40±9.96	318.00±7.30 ^b	212.70±15.56 ^a	218.40±16.00 ^a
Plasma glucose (mmol/L)	8.42±0.82	5.01±0.53 ^{ab}	28.08±1.12 ^a	26.22±0.61 ^{ab}

Values are presented as mean (SD). ^a $p < 0.05$ values are significant compared with non-diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. NC; Normal control, MO; *Moringa oleifera*-treated control rats, DM; Diabetic rats, DM + MO; *Moringa oleifera*-treated diabetic rats.

Diabetic rats injected with STZ showed elevated glucose levels, which is indicative of hyperglycemia, an observation also reported by other authors [57,58]. The potential

hypoglycemic effect of MO is demonstrated by its glucose lowering effect of non-diabetic treated rats. These results are in conformity with other studies [59,60].

Increased kidney size is a sign of acute inflammation and was observed in diabetic rats compared to the non-diabetic and non-MO treated controls in this study (Table 6). Treatment with MO reduced kidney size gain, showing a hypolipidemic effect of MO in the kidney of diabetic rats. Our finding agrees with the findings of previous authors who reported that kidney enlargement may be due to hyperplasia (rapid production of cells leading to enlarged tissue) and hypertrophy (enlargement of cell components) of the kidney [61]. Elevation of oxidative stress levels in the cells consequently leads to cell dysfunction, which is a primary cause of chronic hyperglycemia [62].

4.6.2 Estimation of antioxidant capacity, total polyphenols, flavonoids and flavonols content of *Moringa oleifera* methanol extracts

Although other studies have been conducted on MO, the antioxidant activity of the methanol leaf extract has been reported only in limited extent. This study expanded upon this in a comprehensive antioxidant study (Table 7). The assessment of total antioxidant capacities of methanol extracts of MO was conducted using three complementary assays: Oxygen radical absorbance capacity (ORAC), Ferric reducing antioxidant power (FRAP), trolox equivalence antioxidant capacity (TEAC). The results were estimated as trolox equivalent per gram as ORAC ($3652.14 \pm 113.32 \mu\text{mol TE/L}$), TEAC ($96.09 \pm 1.58 \mu\text{mol TE/L}$) and ascorbic acid equivalent per gram as FRAP ($1736 \pm 3.08 \text{ AAE/L}$). Results showed high antioxidant capacity of MO which is capable of combating reactive species.

Also, the high concentration of total polyphenols, flavonoids and flavonols content were estimated in the methanol extracts of MO. Total polyphenols content of methanol extracts were (2454.00 ± 17.54) mg GAE/L, flavonoids (297.23 ± 30.00) mg QE/L and flavonols (148.70 ± 4.00) mg QE/L.

Table 7: Estimation of antioxidant capacity, total polyphenols, flavonoids and flavonols content of *Moringa oleifera* extracts

Parameters	MO Methanol extract
ORAC $\mu\text{mol TE/L}$	3652.14 \pm 113.32
FRAP $\mu\text{mol AAE/L}$	1736 \pm 3.08
TEAC $\mu\text{mol TE/L}$	96.09 \pm 1.58
Total polyphenols mg GAE/L	2454.02 \pm 17.54
Flavonols mg QE/L	297.23 \pm 30.00
Flavonoids mg QE/L	148.70 \pm 4.00

Values are presented as (mean \pm SD). TE (trolox equivalent), AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), QE (quercetin equivalent).

Antioxidants can help the biological system to defend itself, mop up and repair damages caused by free radicals, and this is done by donating an electron to free radicals to make them stable [38]. Phytochemicals such as total polyphenols, flavonoids, flavonols, vitamins, glutathione, α -tocopherols, β -carotene, and carotenoids are very good, less expensive and non-toxic sources of antioxidant [63,64]. MO has been shown to possess a high level of all these antioxidant activities [65-68]. Increased daily antioxidant consumption can also limit free radical damage. Interestingly, MO is capable of preventing or slowing the oxidation of other molecules generally by trapping free radicals and reducing the development of inflammatory cytokines because of its high phenolic contents [69,70]. In this study, antioxidant activities were determined by measuring concentrations of total polyphenol (2454.02 \pm 17.54 mg GAE/L), flavonols (297.23 \pm 30.00 mg QE/L) and flavonoid (148.70 \pm 4.00 mg QE/L) content in the methanol extract of MO (Table 7). High levels of total polyphenols, flavonols and flavonoids content were reported in MO suggesting its ability to lower oxidative enzymes and reduce cellular damage. This high phenolic content is responsible for its antidiabetic and antioxidant properties.

Antioxidant capacity was used to measure the antioxidant potency and phytochemical concentration of the leaf extract elucidating its free radical scavenging ability [71,72]. In this study, the antioxidant capacity of methanol MO extracts was determined by using oxygen

radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) and trolox equivalence antioxidant capacity (TEAC) prior to its administration in rats (Table 7). Results showed ORAC: (3652±113.32 $\mu\text{mol TE/L}$), FRAP: (1736±3.08 $\mu\text{mol AAE/L}$), TEAC: (96.09±1.58 $\mu\text{mol TE/L}$). This is indicative of a very high radical scavenging capability and the presence of polyphenols revealed the phytochemical activity of MO leaves as seen in Table 7.

4.6.3 Determination of serum total protein, albumin and globulin concentrations

In Table 8, total protein and albumin concentrations significantly decreased ($p < 0.05$) in diabetic rats compared to non-diabetic control. MO treatment of non-diabetic rats elevated ($p < 0.05$) serum concentrations of total protein, albumin and globulin ($p < 0.05$) compared to normal control (NC). Also, a slight increase in total protein and globulin levels were observed in MO-treated diabetic rats in comparison to diabetic control.

Table 8: Serum total protein, albumin and globulin concentrations

Parameters	NC	MO	DM	DM+MO
Total protein g/l	53.86±2.36	70.04±6.05 ^{ab}	44.66±6.92 ^a	50.54±1.76
Albumin g/l	32.50±0.89	39.36±3.03 ^{ab}	27.55±1.17 ^a	29.69±0.94 ^a
Globulin g/l	22.98±2.13	43.34±3.89 ^{ab}	21.21±0.79	26.06±7.68

Values are presented as mean (SD). ^a $p < 0.05$ values are significant compared with non-diabetic control.

^b $p < 0.05$ values are significant compared with diabetic control.

NC; Normal control, MO; *Moringa oleifera*-treated control rats, DM; Diabetic rats, DM + MO; *Moringa oleifera*-treated diabetic rats.

In a diabetic state, loss in blood protein (albumin and globulin) is observed and when excessive, it alters the normal filtering mechanism of the kidneys resulting in the accumulation of toxic wastes. Albumin and globulin serve as transport proteins and biomarkers in disease state [73]. In this study, total protein, albumin and globulin levels were lowered in diabetic groups compared to non-diabetic groups (Table 8). This reduction of protein concentration in the serum possibly due to increased lipid peroxidation and a decrease in the antioxidant defence system. MO administration increased the levels of total protein, albumin and globulin in the serum in comparison with the diabetic control but not significantly suggesting its ability to enhance kidney function after treatment.

4.6.4 Effect of lipid peroxidation and activities of antioxidant enzymes in the kidney

Table 9 shows the effect of lipid peroxidation and activities of antioxidant enzymes in the kidney of rats. Diabetic rats showed significantly ($p < 0.05$) increased Malondialdehyde (MDA) levels when compared to normal control. Subsequent treatment of diabetic rats with MO led to a significant ($p < 0.05$) decrease in MDA levels when compared with diabetic control.

Furthermore, CAT activity significantly ($p < 0.05$) decreased in the kidney of diabetic rats when compared to normoglycemic rats and also significantly increased in diabetic treated rats in comparison to diabetic control. The activity of SOD increased while GPx decreased and total GSH levels remained unchanged in the kidney of untreated diabetic rats when compared to normal control. In diabetic rats, GPx activity decreased significantly treated groups after treatment with MO when compared to normal control while CAT increased after MO treatment as compared to diabetic control.

Table 9: Lipid peroxidation and activities of antioxidant enzymes in the kidney

	NC	MO	DM	DM+MO
MDA ($\mu\text{mol/g}$)	0.48 \pm 0.04	0.43 \pm 0.05 ^b	0.72 \pm 0.12 ^a	0.54 \pm 0.06 ^b
CAT (U/mg protein)	0.42 \pm 0.02	0.47 \pm 0.01 ^b	0.28 \pm 0.06 ^a	0.30 \pm 0.05 ^{ab}
SOD (U/mg protein)	1.01 \pm 0.40	1.35 \pm 0.11 ^{ab}	0.78 \pm 0.30	0.92 \pm 0.0.1
GSHt ($\mu\text{mol/g}$)	2.00 \pm 0.26	1.70 \pm 0.28	1.67 \pm 0.47	1.86 \pm 0.0.24
GPx (U/mg protein)	1.29 \pm 0.33	0.72 \pm 0.23 ^a	1.11 \pm 0.40	0.87 \pm 0.40 ^a

Values are presented as mean (SD). ^a $p < 0.05$ values are significant compared with non-diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control; NC; Normal control, MO; *Moringa oleifera*-treated control rats, DM; Diabetic rats, DM + MO; *Moringa oleifera*-treated diabetic rats.

High concentration of ROS causes damage to structural biomolecules such as proteins, lipids and carbohydrates leading to the inability of the body's defence mechanism in protecting cellular integrity [74]. Free radicals cause lipid peroxidation, and when this happens, the lipids of the cell membrane undergo catabolism leading to tissue damage [75]. In the present study, the levels of MDA- a biomarker of lipid peroxidation, significantly increased in the kidney of diabetic rats when compared to the normal (non-MO treated) control (Table 9). The increase in MDA level in diabetic groups suggests damage to cell membrane lipids, as a result of

increased generation of ROS after STZ-treatment [76]. Subsequent treatment with MO led to a significant ($p < 0.05$) decrease in MDA levels and restoration to near-normalcy. Similarly, Verma et al. [68] reported a reduced MDA levels after treatment of animals with MO. MO exerts its ameliorative effects by reducing oxidative stress, endothelial cell dysfunction, kidney lipid peroxidation and increasing antioxidant capability. These effects may be related to MO's phytochemical composition which appears to commence a recovery process from associated kidney related damage.

Antioxidant enzymes (CAT, SOD, GPx) and non-enzymatic antioxidants (GSH) delay or prevent the oxidation of substrates and prevent ROS-induced oxidative stress [76,77]. SOD represents the first line of defence against oxygen-derived radicals (ROS), as it is responsible for the dismutation of superoxide radicals to H_2O , whereas catalase metabolically removes hydrogen peroxide and hydroxyl radical generation [8]. The synergistic relationship between CAT and SOD against ROS accumulation inactivates peroxy radical and superoxide anion converting them to water and oxygen [25]. GSH is a thiol group-containing molecule, well known for its effective antioxidant property in scavenging hydroxyl radical and singlet oxygen [51]. GPx detoxifies H_2O_2 and lipid peroxides using GSH as substrate.

In the current study, the activity of CAT was significantly reduced while SOD, GSH and were reduced but not significantly in the kidney tissue homogenate of the diabetic control group (Table 9) when compared to normal control, non-diabetic treated and diabetic treated groups. GPx reduced in the diabetic control group in comparison to normal control groups. Following treatment with MO, the activities of CAT, SOD and GSH were enhanced in all MO treated groups, suggesting MO's ability to scavenge and neutralize STZ induced oxidative stress, thus providing a significant recovery in the altered enzyme defence mechanism in the treated groups. This may be related to the presence of terpenoids in MO [78].

4.6.5 Effect of *Moringa oleifera* on kidney histopathology

Histopathological examination of the kidney sections of non-diabetic and diabetic rats revealed the protective effects of MO on the kidney (Figure 16 a-d). Non-diabetic control and non-diabetic treated rats showed no visible lesions and normal glomeruli (Figure a & b). In diabetic control rats, severe interstitial congestion at the cortical area of the kidney was observed. Haemorrhage and congestion of the glomerulus were also observed (Figure c). Diabetic treated rats showed very mild vascular congestion of the glomerulus (Figure d).

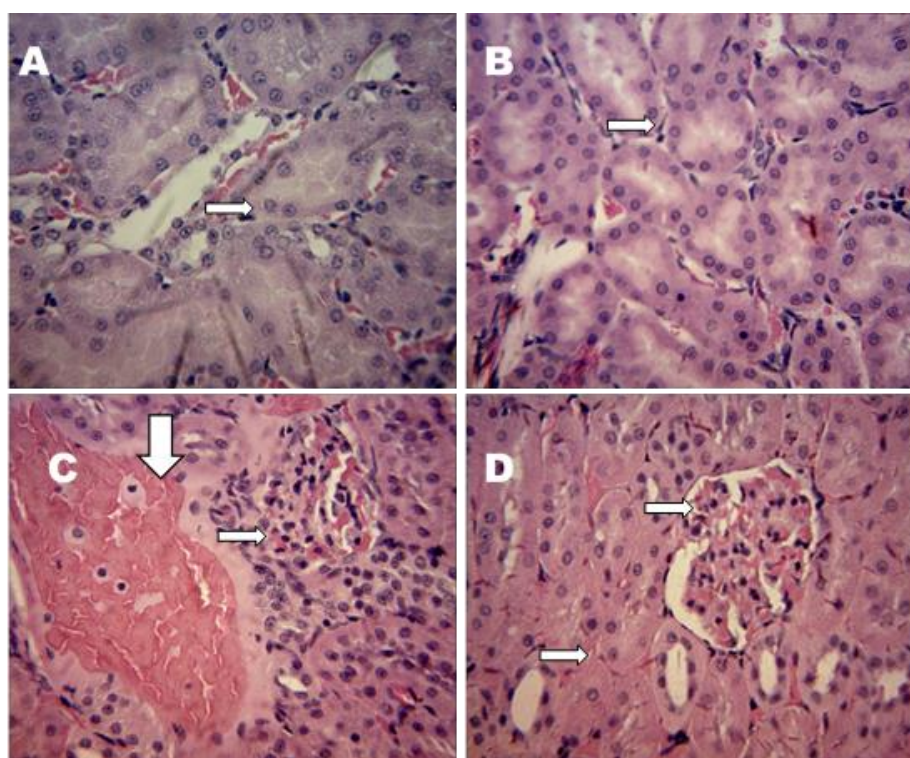


Figure 16: Effect of *Moringa oleifera* on the renal sections (hematoxylin and eosin stained X 400). (a) Control (b) *Moringa* treated non-diabetic rats (c) diabetic control rats and (d) diabetic *Moringa* treated rats.

Microscopic anatomical analysis of the kidney tissue was analysed. Histopathological sections of the kidney of non-diabetic rats indicated normal cell structure. Kidney sections of diabetic rats demonstrated severe renal damage showing interstitial nephritis at the cortical area of the kidney. Glomeruli haemorrhage is associated with diabetes. However, administration of MO showed appreciable improvement to these alterations showing only mild vascular congestion of the glomerulus afterwards, suggesting that diabetic nephropathy was significantly reduced after MO treatment. This is consistent with the findings of Kandasamy and Ashokkumar (2013) who reported significant changes in the pathology of the kidney of diabetic rats after treatment.

4.6.6 Effect of *Moringa oleifera* on Tumour necrosis factor (TNF- α) and Interleukin IL-6 in the kidney

Effect of MO on tumour necrosis factor (TNF- α) and Interleukin IL-6 in the kidney is illustrated in Figure 17. Inflammatory biomarkers TNF- α and IL-6 concentrations in Figures 17 a & b respectively showed increase in the diabetic groups when compared to non-diabetic groups. Diabetic induction significantly ($p < 0.05$) increased levels of IL-6 with no effect on the renal levels of TNF- α . TNF- α and IL-6 levels decreased in the non-diabetic treated rats when compared to normal control. Treatment with MO in the diabetic rats did not lead to a significant reduction in TNF- α and IL-6 concentrations in the kidney.

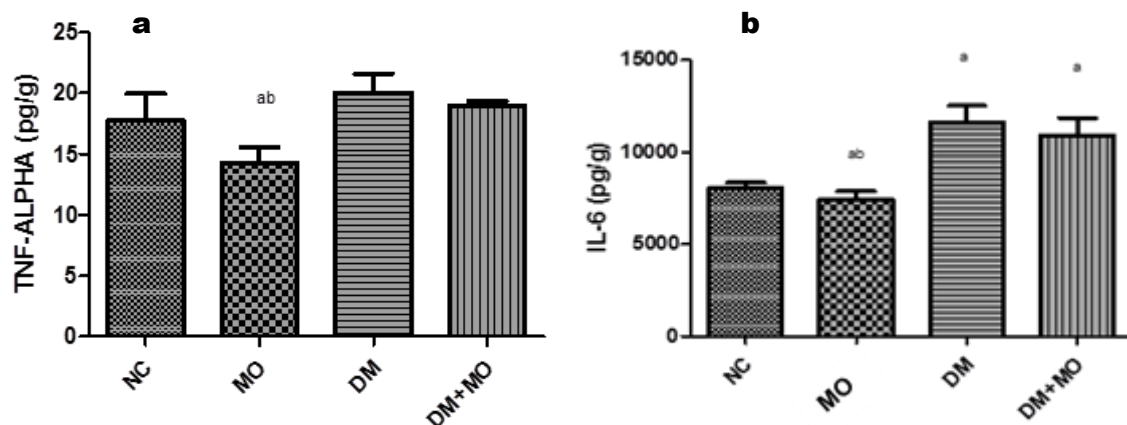


Figure 17: Effect of *Moringa oleifera* on (a) tumour necrosis factor alpha (TNF- α) and (b) interleukin-6 (IL-6) in the kidney. Values are presented as mean (SD). ^a $p < 0.05$ values are significant compared with non-diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control NC; Normal control, MO; *Moringa oleifera*-treated control rats, DM; Diabetic rats, DM + MO; *Moringa oleifera*-treated diabetic rats.

Inflammation in tissues occurs as a response to harmful stimuli or damage to cells. IL-6 and TNF- α cytokines play a crucial role in hyperglycemia-induced kidney injury and are associated with the development of diabetes and with increasing levels in patients with diabetic nephropathy suggesting that these cytokines play significant roles in the pathogenesis of diabetic nephropathy as reported by Navarro et al. [60]. Seca et al. [78] demonstrated in their study the presence of high concentration of inflammatory proteins which caused destruction the pancreatic beta cells, thus affecting insulin secretion.

There is convincing experimental and clinical evidence which indicate an increase in the generation of ROS and systemic markers of inflammation in both types of diabetes which are in line with this study [60, 79,80]. Induced-diabetic rats showed an increased level of IL-6 and TNF- α which did not reduce with concurrent treatment with MO.

The results showed that MO extracts have the potency to counteract the formation of free radicals and other reactive species generated in the kidney but not in a diabetic state thereby decreasing oxidative stress, inflammation and preventing cellular damage. The observed decrease in the inflammatory cytokines in the non-diabetic rats showed retardation in the onset of diabetic nephropathy [81, 82].

4.7 Conclusion

Hyperglycemia was successfully induced in the animal model which was confirmed in the blood and kidney biomarkers. Oxidative stress, inflammation and nephropathy were observed in diabetic groups while treatment with methanol extract of MO ameliorated the effect. This suggests that MO has displayed antioxidant capacity due to its high polyphenols, flavonoids and flavonols content. From our studies, MO was also able to enhance the antioxidant status and reduce lipid peroxidation showing that MO has the potential to be used as an antidiabetic agent in the treatment and management of diabetes. Further studies are recommended in isolating polyphenolics compounds in pure form so as to establish the mechanisms of action and the structure elucidation.

Declarations

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Authors' contributions

EO Performed the experimental work, analysed and wrote the first draft of the manuscript. GA supervised the study and reviewed manuscript. OO designed the work, supervised the entire study and critically reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The data supporting the conclusions in this study will be made available by contacting the corresponding author.

Competing interests

The authors declare no conflict of interest.

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CHAPTER FIVE

5 **Hepatoprotective activity of *Moringa oleifera* in diabetes-induced liver damage in male Wistar rats**

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ABSTRACT

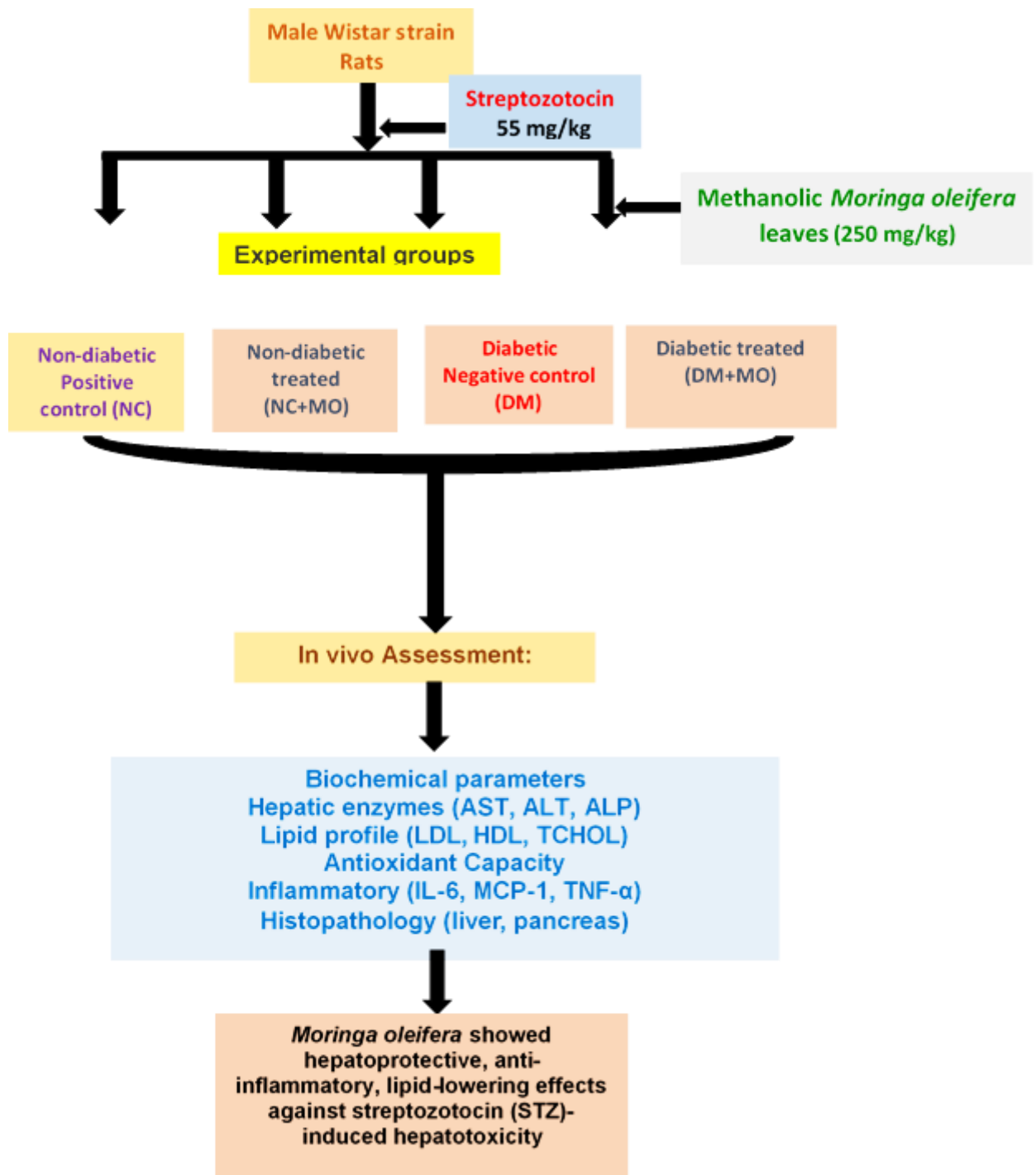
Background: The number of individuals with diabetes is increasing daily, and diabetes is presently estimated to affect about 422 million adults worldwide. Conventional drugs used to treat diabetes are not without severe side effects, accessibility, and affordability. This study elucidates the potential effects of *Moringa oleifera* (MO) leaves extract to manage and treat diabetes induced in male Wistar rats.

Materials and Methods: Adult male Wistar rats were randomly divided into four groups ($n = 12/\text{group}$): NC- nondiabetic rats (positive control), MO -nondiabetic-treated rats, DM -diabetic rats (negative control), DM + MO-diabetic-treated rats. Hepatic enzymes and biochemical parameters, antioxidant capacity and inflammatory cytokine levels were assessed. Levels of low-density lipoprotein, high-density lipoprotein, and total cholesterol were evaluated. Liver and pancreas histopathology were evaluated.

Results: Oral administration of methanol extract of MO (250 mg/kg) to diabetic rats for 42 days showed a significant reduction in hepatic enzyme markers and normalized lipid profile parameters in the serum compared to normal control group. Treatment with MO also increased the level of antioxidant capacity and a reduction in the liver inflammatory markers (IL-6, TNF- α and chemokine MCP-1) in diabetic rats. Histology sections of the liver and pancreas tissues showed protective effects of MO in treated rats.

Conclusions: MO showed hepatoprotective, anti-inflammatory, and lipid-lowering effects against streptozotocin-induced hepatotoxicity. Histological section demonstrated specific alterations in the liver of the diabetic and nondiabetic male Wistar rats while MO treatment revealed a reversal of liver alterations.

Key words: Diabetes, hepatic enzymes, inflammatory cytokines, lipid profile, *Moringa oleifera*, streptozotocin



5.1 INTRODUCTION

The number of individuals with diabetes is increasing daily and is presently estimated to affect over 422 million adults worldwide [1]. Diabetes is associated with liver cirrhosis, inflammation, apoptosis, microvascular and macrovascular complications [2]. This is linked with a significant increase in inflammatory cytokines; C-reactive protein, interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrotic factor-alpha (TNF- α), and chemokine monocyte chemotactic protein-1 (MCP-1). Reports have shown hyperglycemic effects from uncontrolled hepatic glucose output and reduced glucose uptake by skeletal muscle coupled with reduced glycogen synthesis [3].

In endogenous systems, normal metabolic processes generate highly reactive compounds called free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS can be introduced by other sources [4]. These molecules possess unpaired electrons in the outer shell and are very unstable, hence becoming highly reactive and adversely altering cellular macromolecules [5]. High level of these reactive compounds causes oxidative damage, oxidative stress and deleterious effects thus leading to increased risk of developing chronic diseases or enhancing the pathogenesis/complications of chronic diseases such as DM [6,7]. Free radicals have been implicated in a number of diseases such as diabetes, cancer, ageing, cardiovascular diseases and neurodegenerative diseases [8,9].

These free radicals also impair the antioxidant defense system leading to high secretion of inflammatory cytokines and cell proliferation [10,11]. The antioxidant defense system comprises endogenous sources such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and reduced glutathione. Other exogenous antioxidant sources include vitamin C, E, A, carotenoids, phenols and glucosinolates which are capable of protecting the system from free radicals generation and stabilizing radicals before they attack cells [12,13]. Furthermore, when free radicals generation exceeds the protective effects of enzymatic and non-enzymatic antioxidants, oxidative damage accumulates promoting complications in various chronic diseases [14]. Moreover, there is increasing evidence from experimental and clinical studies indicating oxidative stress as a major player in diabetic pathophysiology leading to

dyslipidemia, impaired glucose tolerance, β -cell dysfunction, and ultimately resulting in liver and kidney malfunction [15,16].

Increased intake of antioxidant-rich natural products can help in reducing the risk of free radical-related health challenges [12,17]. Presently, there has been appreciable interest in various therapeutic remedies including medicinal plants in the management of chronic diseases and some of them have displayed minimal or negligible side effects [18]. Conventional drugs, metformin and glimepiride, are used to treat diabetes but with severe side effects, lack of accessibility and affordability [19]. Unconventional therapy is known to play significant roles in treating and managing diabetes with minimal side effects coupled with its easy access and affordability [20]. In the diabetic state, there is increased generation ROS which triggers a chain of reaction leading to the peroxidation of lipids, lipoprotein modifications and several cellular mutations of biomolecules [21,22]. These effects lead to oxidative stress which is an important risk factor in the pathogenesis of cardiovascular and chronic diseases such as diabetic and associated complications [23].

Moringa oleifera (MO) is the most cultivated species of the Moringaceae family [24]. It has been reported in folk medicine for its use in the treatment and management of diabetes [25]. Interestingly, studies have shown that MO leaves are rich sources of antioxidant due to the presence of various phytochemicals such as polyphenolics, carotenoids, α -tocopherol, ascorbic acid and several amino acids [26-28]. The bioavailability of these metabolites in MO has been directly linked to various biological profiles especially its scavenging activity which helps to ameliorate the damaging effects caused by oxidative stress in diabetic rats [29-31]. Other notable metabolites with medicinal importance from the extracts of MO leaves include glycosides such as niazirin, niazirin, niaziminin A and B, 4-(α -1-rhamnopyranosyloxy)-benzylglucosinolate [32-35]. Scientists have reported the potency of various parts of this plant in animal models for their medicinal properties such as anti-diabetic, antifertility, anticonvulsant, antiallergic and antihelminthic activities [36-39]. These biological and pharmacological activities may help to reduce cell death and cell proliferation [38]. Sreelatha et al. [40] reported anti-proliferative effect

of MO leaf extract on human cancer cells. MO has also been reported to elicit hepatoprotective properties in rats [41,42]. Previous scientific reports on MO in the treatment of diabetes are limited in scope and do not extensively elucidate its action in the liver and erythrocytes, hence the need for this study to examine specific alterations in the liver, erythrocytes and elucidate the hepatoprotective, anti-inflammatory and lipid lowering effects of MO in diabetic and non-diabetic male Wistar rats.

5.2 MATERIAL AND METHODS

5.2.1 *Animal care*

Healthy male adult Wistar strain rats weighing between 200-250 g were purchased from Charles River (Margate, UK) and housed at the Stellenbosch University Animal Research Facility, Stellenbosch University (Tygerberg Campus, South Africa) where animal treatment took place. For the duration of the study, the rats were housed in stainless steel cages of five rats per cage in a temperature controlled animal facility of 22 ± 2 °C and humidity 55 ± 5 % with alternating 12 h light / 12 h dark cycles. The rats had access to fresh water and standard rat chow (SRC) from Aquanuro, Malmesbury, South Africa *ad libitum*.

5.2.2 *Ethical approval*

The study received approval from the Faculty of Health and Wellness Sciences, Research Ethics Committee of Cape Peninsula University of Technology, South Africa (CPUT/HW-REC 2014/AO8). All animals in the study received humane care according to the principles of laboratory animal care of the National Institutes of Health Guide for the care and use of laboratory animals of the National Academy of Science (NAS) and published by the National Institutes of Health (publication no. 80-23, revised 1978).

5.2.3 *Plant material*

MO fresh leaves were collected from the botanical garden and identified and authenticated by Mr A Adeyemo of Forestry Research Institute of Nigeria (FRIN). A voucher specimen was deposited in the herbarium with specimen number FHI-110287. MO leaves were washed with water, dried

and pulverized. 1 kg of leaf powder was extracted with n-hexane and dried marc was extracted with methanol at 45 °C for 24 hours. The filtrate was filtered using Whatman no. 1 filter paper and then evaporated in a rotatory evaporator under reduced pressure at 35 °C. The methanol extracts were stored at -4 °C until use.

5.2.4 Induction of diabetes

Induction of diabetes in rats was done by a single intraperitoneal injection of freshly prepared streptozotocin (55 mg/kg bd. wt., Sigma, Munich, Germany) in citrate buffer (0.1 M at pH 4.5) according to the method of Jaiswal et al. [31]. Blood samples were collected from the rat tail and fasting blood glucose level was measured using a glucometer (Accu-Check, Roche, Germany) and test strip. Only rats with glucose levels of ≥ 18 mmol/L were considered diabetic and included in the study [31]. MO administration commenced on the fourth day after the rats were confirmed diabetic and this was considered the first day of treatment. The rats were randomly assigned into four (4) groups of twelve rats (n = 12).

5.3 Experimental protocol

Forty-eight rats were randomly divided into four groups as follows:

Group I: (NC) consisting of non-diabetic rats, which were treated with vehicle and SRC only.

Group II: (MO) non-diabetic treated rats received only MO extract at a dose of 250 mg/kg and SRC.

Group III: (DM) were diabetic and received the vehicle and SRC without receiving the plant extract.

Group IV: (DM+MO) diabetic treated rats received only MO extract at a dose of 250 mg/kg and SRC.

Following the 250 mg/kg dose of MO extract administered by Efiog et al. (2013) in their research, the same dose was found to be most suitable for this study. MO methanol extracts were dissolved in the vehicle and administered orally via oral gavage daily for six (6) weeks to the treated groups while positive control groups and negative control groups received the vehicle. All rats received SRC daily for the duration of the study. The rats were weighed at the beginning and at the end of the experiment. At the end of study period, animals were weighed, fasted overnight and

anaesthetized with 60 mg/kg sodium pentobarbital intraperitoneal injection in order to ensure rapid and painless death of rats.

5.3.1 Serum preparation

Whole blood was collected from the rat's abdominal aorta into a serum clot activator tube and plasma into a lithium heparin plasma separator tubes. Serum and plasma was prepared by centrifugation at 4000 g for 10 minutes at 4 °C and stored at -80 °C for biochemical analysis

5.3.2 Organ preparation

Liver and pancreas were excised, rinsed in phosphate-buffered saline and weighed, after which the liver was homogenized in ice-cold phosphate buffered saline (PBS, 50 mM pH 7.5) to release endogenous enzymes from cells. Homogenates were centrifuged at 15000 rpm for 10 minutes at 4 °C, then aliquoted and stored at -80 °C for biochemical analysis.

5.3.3 Preparation of erythrocytes

Blood was drawn from the abdominal aorta of the animals into tubes and plasma was separated via centrifugation at 4000 g for 10 minutes at 4°C. The erythrocytes were isolated from the plasma by centrifugation for 10 minutes at 4000 g at 4°C to remove the buffy coat. The erythrocytes were washed thrice with ice cold phosphate-buffered saline (PBS) and centrifuged. The erythrocytes were resuspended in the PBS at 1:9 dilutions and used for antioxidant indices and lipid peroxidation.

5.3.4 Determination of weight parameters, glycated haemoglobin and glucose level

Liver and pancreas were weighed and relative weights were calculated by dividing the liver and pancreas weight by total body weight of each rat respectively after termination. Glycated haemoglobin (HbA1c) level in the erythrocytes and serum glucose were measured using assay kit from Randox Laboratories Limited (Crumlin, United Kingdom) on an automated Randox Daytona from Randox Laboratories.

5.3.5 Determination of plasma antioxidant capacity

Trolox equivalence antioxidant capacity (TEAC): TEAC was analysed in the plasma using the method described by Re et al. [43]. Trolox was used as standard. *Ferric reducing antioxidant power* (FRAP): FRAP plasma was assayed using the protocol adapted from Benzie & Strain [44]. *Oxygen radical absorbance capacity* (ORAC): The method of Ou et al. [45] was used to determine the oxygen radical absorbance capacity (ORAC) in plasma.

5.3.6 Determination of antioxidant enzyme activities and reduced glutathione in the liver and erythrocytes

Superoxide Dismutase (SOD): SOD activity was assessed in the liver homogenate and erythrocytes using a Multiskan plate Thermo Electron Corporation reader (Beverly, MA, USA) with methods described by Crosti et al. [46].

Catalase (CAT): CAT activity was determined spectrophotometrically at 240 nm in the liver homogenate and erythrocytes using the method of Aebi [47].

Total glutathione (GSH): The level of total glutathione GSH in the liver homogenate and erythrocytes was estimated following methods described by Asensi et al. [48].

Glutathione Peroxidase (GPx): Level of GPx activity in the liver homogenate and erythrocytes was determined spectrophotometrically at 340 nm using the method of Elleby and Bredesen [49].

5.3.7 Analysis of liver enzymes activity

Liver enzyme activities namely aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) were determined. AST activity is based on the reaction of α -ketoglutarate with L-aspartate to form oxaloacetate and L-glutamate. The oxaloacetate formed further reacts with NADH to form L-malate and NAD^+ . ALT activity was estimated kinetically based on the reaction of α -ketoglutarate and L-alanine catalyzed by ALT to form L-glutamate and pyruvate. The indicator reaction used pyruvate for the kinetic estimation of NADH consumed. The pyruvate formed further reacts with NADH to form L-lactate. Measurement of ALP is based on the reaction of p-nitrophenyl phosphate which was hydrolysed by alkaline phosphatase in the serum in the presence of magnesium ion to yield p-nitrophenol (yellow). The colour intensity was

proportional to the activity of alkaline phosphatase in the serum. The complex formed was measured using assay kit from Randox Laboratories Limited (Crumlin, United Kingdom) on an automated Randox Daytona from Randox Laboratories. All protocol followed according to the manufacturer's manual.

5.3.8 Determination of lipid profile

Lipid profile such as low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol (CHOL) was analysed in the serum. All assays were analysed using diagnostic kits (Randox Crumlin, United Kingdom) using Randox Daytona automated analyzer (Randox Laboratories Limited, Crumlin, United Kingdom). Protocols were followed according to the manufacturer's standard operating procedures.

5.3.9 Analysis of Inflammatory cytokines in the liver

The concentration of inflammatory cytokines interleukin-6 (IL-6), monocyte chemotactic protein (MCP)-1 and tumour necrotic factor (TNF- α) were evaluated in the liver homogenates using MILLIPLEX[®] MAP rat cytokine magnetic bead-based Luminex kit (Merck Millipore, Billerica, MA, USA). Assays were performed strictly according to the manufacturer's protocol (Merck Millipore) and read on the Bio-plex[®] platform (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Plex Manager[™] software, version 6.0 was used for bead acquisition and analysis of median fluorescent intensities.

5.3.10 Histopathological analysis

The liver and pancreas were processed by embedding in paraffin and sectioned to 5 μ m thickness. After deparaffinization, the slides were stained routinely with haematoxylin and eosin (H & E). The examination of stained slides and photomicrography of slides were performed by a pathologist who was blinded to the control or treatment groups in the study.

5.4 Statistical analysis

Values are represented as the mean and standard deviation (mean \pm SD). GraphPad Prisms version 5.0 for Windows (San Diego, CA, USA) was used to analyse data using ANOVA with Bonferroni's multiple comparison tests. p values < 0.05 were considered significant.

5.5 RESULTS

5.5.1 Effect of *Moringa oleifera* on weight parameters and serum glucose level

The effect of MO on weight parameters and serum blood glucose level is shown in Table 10. Body weight of rats was taken before and at the end of the experiment with a change in body weight observed in all experimental groups. Diabetic rats showed a significant decrease ($p < 0.05$) in body weight when compared to non-diabetic control. It can be seen that the liver weight and the relative liver weight of animals injected with STZ were heavier compared to non-diabetic control rats and non-diabetic treated rats respectively. However, significant ($p < 0.05$) decrease in liver weight and relative liver weight of diabetic rats was observed after MO treatment when compared to diabetic control group. Treatment with MO in diabetic rats showed significant ($p < 0.05$) decrease in pancreas and relative pancreas weight. Elevated serum glucose level decreased significantly ($p < 0.05$) in groups administered with MO when compared to diabetic control. HbA1c level increased significantly ($p < 0.05$) in diabetic rats, this level reduced significantly ($p < 0.05$) after treatment with MO.

Table 10: Body weight, organ weights, serum glucose level and glycated haemoglobin

Parameters	Control NC	<i>Moringa</i> MO	Diabetic DM	Diabetic+ <i>Moringa</i> DM+MO
<i>Initial body wt. (g)</i>	229.30 \pm 13.61	230.30 \pm 9.38	229.00 \pm 14.73	230.90 \pm 10.58
<i>Final body wt. (g)</i>	313.40 \pm 9.96	318.00 \pm 7.30 ^b	212.70 \pm 15.56 ^a	218.40 \pm 16.00 ^a
<i>Change in body wt. (%)</i>	32.00 %	28.11%	-6.70%	-8.16%
<i>Liver wt (g)</i>	8.50 \pm 0.46	7.48 \pm 0.43 ^{ab}	9.35 \pm 0.68 ^a	8.49 \pm 0.47 ^b
<i>Relative liver wt(g/100 g)</i>	2.62 \pm 0.10	2.45 \pm 0.10 ^{ab}	4.30 \pm 0.32 ^a	3.98 \pm 0.10 ^{ab}
<i>Pancreas wt. (g)</i>	1.13 \pm 0.39	0.87 \pm 0.19 ^b	0.5244 \pm 0.08 ^a	0.6333 \pm 0.11 ^a
<i>Relative pancreas wt. (g/100g)</i>	0.30 \pm 0.05	0.25 \pm 0.02 ^a	0.25 \pm 0.03 ^a	0.27 \pm 0.03
<i>Blood glucose (mmol/L)</i>	7.25 \pm 1.27	5.38 \pm 0.96 ^b	30.96 \pm 2.11 ^a	27.61 \pm 1.77 ^{ab}
<i>HbA1c (g/dl)</i>	2.31 \pm 0.30	2.50 \pm 0.80 ^b	8.71 \pm 1.00 ^a	6.92 \pm 0.40 ^{ab}

Values represent mean (S.D) of 12 animals. ^a $p < 0.05$ values are significant compared with non-diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. **NC**; non-diabetic rats (positive control), **MO**; non-diabetic treated rats, **DM**; diabetic rats (negative control), **DM + MO**; diabetic treated rats. HBA1c (glycated haemoglobin).

5.5.2 Effect of *Moringa oleifera* on plasma antioxidant level

Trolox equivalence antioxidant capacity (TEAC), Ferric reducing antioxidant power (FRAP) and Oxygen radical absorbance capacity (ORAC) content were assayed in the plasma as shown in Table 11. Plasma antioxidant status was assessed by the above-mentioned assays and antioxidant capacity decreased significantly ($p < 0.05$) in diabetic groups when compared to the non-diabetic control. However, following MO treatment, a significant ($p < 0.05$) increase in antioxidant capacity of non-diabetic treated rats was reported and no difference in the diabetic treated groups when compared to the diabetic control.

Table 11: Plasma antioxidant capacity in *Moringa oleifera* treated rats

Parameters	NC	MO	DM	DM+MO
TEAC (nmol TE/L)	96.21±38.67	98.55±34.65 ^b	115.90±26.00 ^a	122.10±21.17 ^a
FRAP(μmolAAE/L)	196.10±8.16	235.70±5.44 ^{ab}	131.00±18.48 ^a	146.00±10.25 ^a
ORAC(μmol TE/L)	217.60±5.97	234.20±6.77 ^b	163.30±9.45 ^a	175.30±6.51 ^a

Values represents mean (S.D) of 12 animals. ^a $p < 0.05$ values are significant compared with non- diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. **NC**; non-diabetic rats (positive control), **MO**; non-diabetic treated rats, **DM**; diabetic rats, **DM + MO**; diabetic treated rats.

5.5.3 Effect of *Moringa oleifera* on liver antioxidant enzymes and reduced glutathione concentration

Table 12 shows the antioxidant enzyme activities in the liver of non-diabetic and diabetic rats. SOD, CAT activities and GSH decreased significantly ($p < 0.05$) in diabetic groups when compared to non-diabetic groups. However, after administration of MO to diabetic rats, CAT activities and GSH increased significantly when compared to the diabetic control rats and SOD increased but not significantly ($p < 0.05$) compared to diabetic control. Treatment of non-diabetic rats with MO showed significant ($p < 0.05$) increase in SOD activity and GSH concentration while CAT decreased when compared to diabetic treated rats. GPx activity increased significantly ($p < 0.05$) in diabetic rats and non-diabetic groups after MO treatment when compared to normal control and diabetic control groups.

Table 12: Liver antioxidant enzyme activities and glutathione concentration

<i>Parameters</i>	NC	MO	DM	DM+MO
SOD (U/μg protein)	8.61±1.14	9.17±1.25 ^b	5.20±0.56 ^a	5.47±0.19 ^a
CAT (U/mg protein)	0.46±0.09	0.42±0.05 ^b	0.10±0.01 ^a	0.39±0.02 ^b
GPx (U/mg protein)	0.60±0.19	0.50±0.16 ^b	1.28±0.24 ^a	1.52±0.27 ^{ab}
GSH (μmol/g)	80.27±6.59	108.40±13.31 ^{ab}	22.10±0.50 ^a	29.14±1.69 ^{ab}

Values represents mean (S.D) of 12 animals. ^a $p < 0.05$ values are significant compared with non- diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. **NC**; non-diabetic rats (positive control), **MO**; non-diabetic treated rats, **DM**; diabetic rats, **DM + MO**; diabetic treated rats.

5.5.4 Effects of *Moringa oleifera* on erythrocytes antioxidant enzyme activities and reduced glutathione concentration

Effects of MO on erythrocytes antioxidant enzyme activities, reduced glutathione and glycated haemoglobin (HbA1c) level is shown in Table 13. SOD activities reduced significantly ($p < 0.05$) in diabetic rats and significantly increased ($p < 0.05$) after MO treatment as compared to diabetic control. CAT activities significantly ($p < 0.05$) decreased in diabetic rats when compared to normal control. Similarly, CAT activities increased in diabetic treated rats after MO administration but not significantly when compared to diabetic control. GSH concentration increased significantly ($p < 0.05$) in diabetic treated groups when compared to non-diabetic groups. GPx activity also decreased significantly ($p < 0.05$) in diabetic rats when compared to normal control with an observable increase after MO treatment.

Table 13: Erythrocytes antioxidant enzymes activities and glutathione

<i>Parameters</i>	NC	MO	DM	DM+MO
SOD (U/μg protein)	7.05±1.00	8.77±0.75 ^{ab}	1.78±1.40 ^a	4.00±0.74 ^{ab}
CAT (U/μg protein)	216.6.80±25.20	194.60±27.67 ^a	152.50±40.50 ^a	185.40±50.55 ^a
GSH (μmol/μg)	219.20±5.21	183.00±2.22.06 ^b	327.20±32.48 ^a	289.80±18.74 ^a
GPx (nmol/min/μgprotein)	2.85±0.33	2.46±0.81 ^c	1.04±0.37 ^a	1.47±0.45 ^a

Values represents mean (S.D) of 12 animals. ^a $p < 0.05$ values are significant compared with non- diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. **NC**; non-diabetic rats (positive control), **MO**; non-diabetic treated rats, **DM**; diabetic rats, **DM + MO**; diabetic treated rats.

5.5.5 Hepatic enzyme activities in non-diabetic rats and diabetic rats

Table 14 shows the activities of liver enzymes in non-diabetic rats and diabetic rats. From the results, a significant elevation in the activities of AST, ALT and ALP in diabetic groups was observed when compared to non-diabetic rats (positive control). Treatment with MO led to a reduction in the activities of hepatic enzymes in all treated groups when compared to normal control.

Table 14: Hepatic enzyme activities in non-diabetic rats and diabetic rats

Parameters	NC	MO	DM	DM+MO
AST(U/L)	195.70±9.02	171.33±5.03 ^{ab}	270.30±9.29 ^a	240.00±13.89 ^{ab}
ALT(U/L)	48.50±10.19	34.00±8.08 ^b	123.3±33.13 ^a	100.00±24.49 ^a
ALP(U/L)	79.71±10.44	61.29±8.10 ^{ab}	345.57±47.12 ^a	190.60±27.66 ^{ab}

Values represents mean (S.D) of 12 animals. ^a $p < 0.05$ values are significant compared with non- diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. **NC**; non-diabetic rats (positive control), **MO**; non-diabetic treated rats, **DM**; diabetic rats, **DM + MO**; diabetic treated rats

5.5.6 Effect of *Moringa oleifera* on lipid profile and antioxidant capacity in the liver of rats.

Table 15 illustrates the effects of MO on low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol (CHOL) and antioxidant capacity in the liver of rats. Induction of diabetes with STZ elevated the levels of LDL and CHOL in diabetic rats (negative control) when compared to non-diabetic rats (positive control). However, after treatment of non-diabetic rats and diabetic rats with MO, LDL and CHOL concentration reduced significantly compared to diabetic rats (negative control). HDL level increased significantly in non-diabetic treated rats compared to non-diabetic and non-treated rats (positive control). There was no significant elevation ($p > 0.05$) in the liver antioxidant capacity (ORAC) of MO treated diabetic rats compared to the negative control but significantly increased ($p < 0.05$) in non-diabetic treated rats when compared to the positive control.

Table 15: Lipid profile and antioxidant capacity in the liver of non-diabetic and diabetic rats

Parameters	NC	MO	DM	DM+MO
LDL(mg/dl)	0.10±0.03	0.06±0.02 ^b	0.14±0.07	0.05±0.02 ^b
HDL (mg/dl)	0.65±0.01	0.73±0.04 ^a	0.63±0.06	0.62±0.05
CHOL (mg/dl)	1.60±0.14	1.36±0.28 ^b	1.92±0.27	1.26±0.19 ^b
ORAC (μmolTE/g)	13.48±0.76	14.87±0.52 ^b	11.84±0.95	12.49±1.12

Values represents mean (S.D) of 12 animals. ^a $p < 0.05$ values are significant compared with non- diabetic control. ^b $p < 0.05$ values are significant compared with diabetic control. **NC**; non-diabetic rats (positive control), **MO**; non-diabetic treated rats, **DM**; diabetic rats, **DM + MO**; diabetic treated rats.

5.5.7 *Moringa oleifera*'s effect on inflammatory biomarkers in the liver of diabetic and non-diabetic rats.

Figure 18 a-c illustrates MO protective effect on inflammatory biomarkers in the liver of diabetic and non-diabetic rats. Concentrations of inflammatory cytokines IL-6, TNF-α and chemokine MCP-1 were elevated in diabetic groups. MO's treatment reduced significantly ($p < 0.05$) inflammatory cytokines in the liver of diabetic rats.

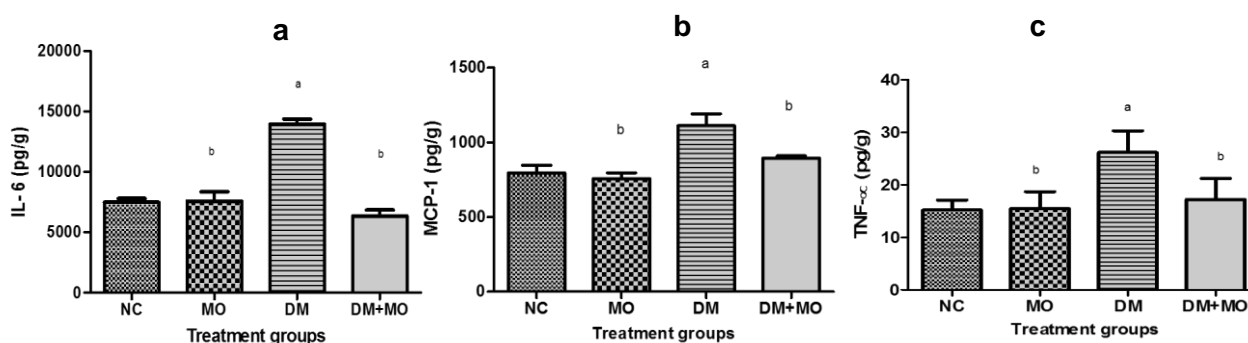


Figure 18: Effect of *Moringa oleifera* on hepatic inflammatory biomarkers (a) Interleukin-6 (IL-6) (b) Monocyte chemotactic protein (MCP-1) (c) Tumour necrotic factor (TNF-α) in diabetic and non-diabetic rats. Each bar represents mean (S.D) of 12 rats. ^a $p < 0.05$ values are significant compared with non- diabetic control (NC). ^b $p < 0.05$ values are significant compared with diabetic control (DM). NC; non-diabetic rats, MO; non-diabetic treated rats, DM; diabetic rats (negative control), DM + MO; diabetic treated rats.

5.5.8 *Histopathological effect of Moringa oleifera on the hepatic structure of rats*

Histopathological liver specimens in non-diabetic treated rats showed no visible lesions when compared to non-diabetic control (Figure 19a & b). In diabetic rats, liver pathology revealed severe

hepatocyte necrosis (1) at the centrilobular zone with a large focus of massive haemorrhage into the liver parenchyma showing undefined boundaries (2) and degenerating nucleus (3) when compared to the diabetic treated rats as shown in Figure 19c. After treating diabetic rats with MO, liver section showed moderate portal congestion and very mild periportal cellular reaction indicating an improvement (Figure 19d).

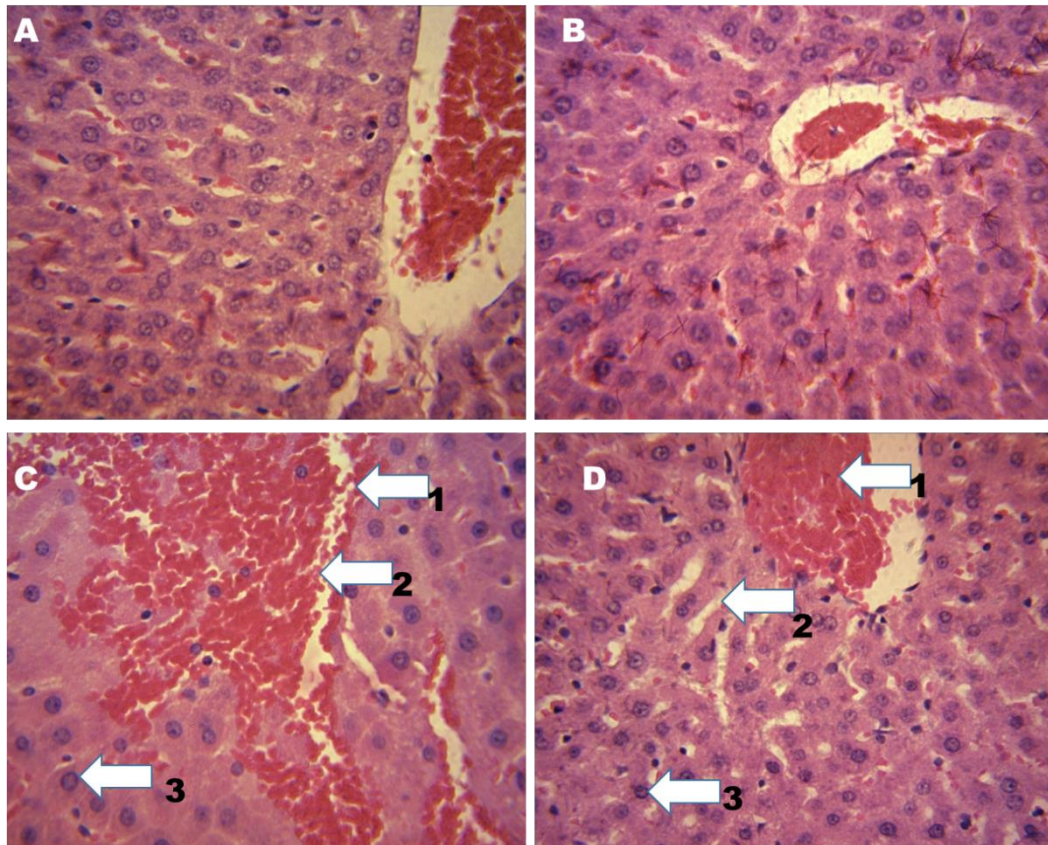


Figure 19: Histopathological effect of *Moringa oleifera* on haematoxylin and eosin stained liver sections of non-diabetic and diabetic rats, (H&E stain 400X). (a) Non-diabetic control rats (b) Non-diabetic treated rats (c) Diabetic control rats and (d) Diabetic treated rats

5.5.9 Effect of *Moringa oleifera* on histopathological sections of the pancreas of non-diabetic and diabetic rats

Pancreatic histology sections of non-diabetic and diabetic rats are shown in Figure 20 (a-d) below. Figure 20 a & b showed sections of non-diabetic control and non-diabetic MO treated rats respectively while Figure 20 c & d showed sections of diabetic and diabetic MO treated rats respectively. From the slides, no visible lesions were observed in non-diabetic MO treated rats (Figure b) when compared to the non-diabetic control rats (Figure a). In contrast, diabetic sections (Figure c) showed severe congestion of the major interstitial vessels thickening of the interstitial vessel and degenerating islets (1) when compared to non- diabetic control rats, After treatment of diabetic rats (Figure d), pancreatic sections of diabetic rats revealed slightly prominent interstitial connective tissues (an improvement) (2) when compared to diabetic control rats (Figure c).

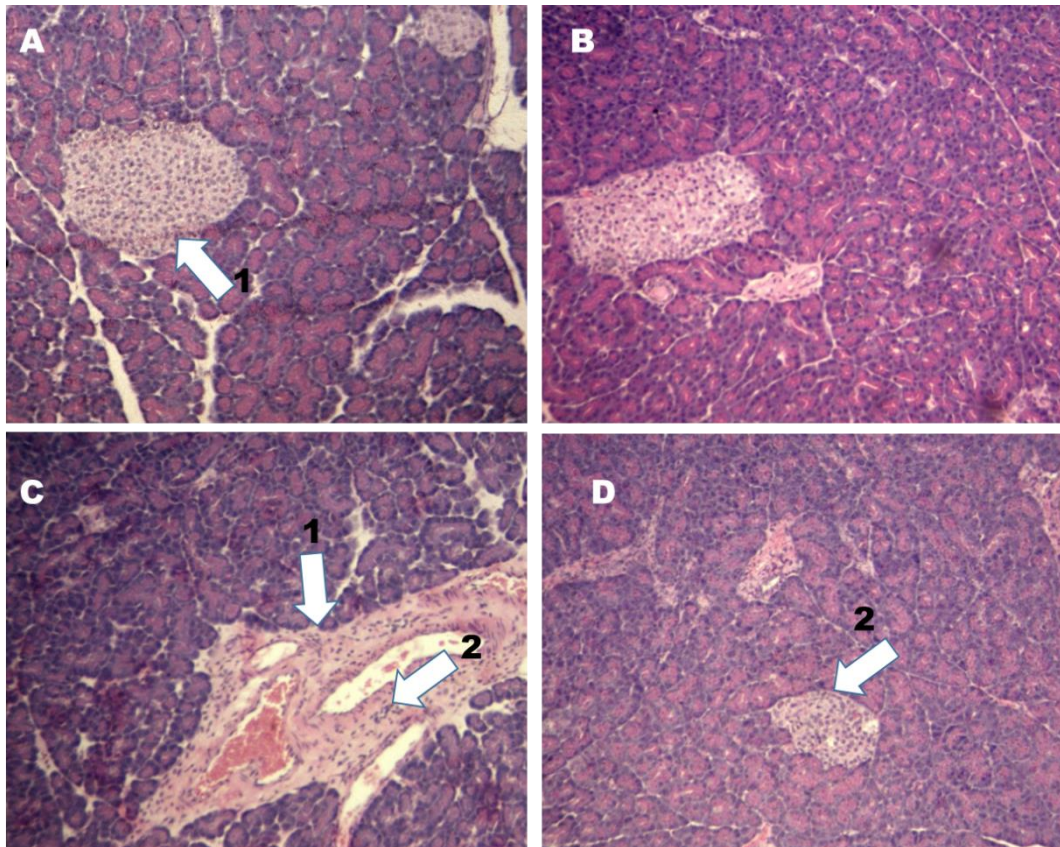


Figure 20: Photomicrograph pancreatic section of non-diabetic and diabetic rats treated with *Moringa oleifera* (haematoxylin and eosin stained, magnification X100). (a) Non-diabetic control rats (b) Non-diabetic treated rats (c) Diabetic control group and (d) Diabetic treated rats

5.6 DISCUSSION

The liver plays a vital role in the regulation of carbohydrate metabolism. It supplies glucose to other organs that require glucose as an energy source and is one of the organs affected by diabetes. Attention has recently been focused on the use of therapeutic agents from plant origin in the treatment and management of diabetes considering the severe side effects of contemporary anti-diabetic drugs [50]. In this study, diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) which caused hepatotoxicity and damage in the liver of diabetic rats. Medicinal plants including MO, have attracted attention for their therapeutic properties and diabetes management. The hepatoprotective, anti-hyperlipidemic and anti-inflammatory action of MO and its ability to modulate hepatic enzymes and biochemical parameters were evaluated in diabetic-induced rats.

Glucose homeostasis (balance between insulin and glucagon to maintain a stable blood glucose level) is essential for the utilization of glucose by the liver, muscles and adipose tissues [51]. Excess glucose concentration causes hyperglycemia and liver damage. Antidiabetic and glucose lowering effects of MO has been reported [52]. In this study, the diabetic-induced condition led to an increased serum glucose level (hyperglycemia). MO treatment significantly reduced glucose level which indicated an improvement in impaired glucose metabolism. MO has demonstrated strong glucose lowering effect and can be used as an antidiabetic component [53].

Final body weight of diabetic treated rats decreased significantly when compared to non-diabetic treated group. Liver weight and relative liver weight increased in non-diabetic treated rats as compared to non-diabetic groups. Liver of diabetic rats experienced significantly increased in size a possible indication of hepatomegaly resulting from the enlargement of tubular cell lining and fatty infiltration [54], which reduced after MO treatment. Reduction in body weight of diabetic rats may be the resultant effect of free radical generation from of STZ-induction [55]. Persistent hyperglycemia may lead to the production of free radicals which attack insulin-producing cells (beta cells) leading to glucose deprivation, promoting protein break down (weight loss) to generate energy [56].

Furthermore, treatment of diabetic rats with MO showed a significant decrease in pancreatic weights and relative pancreas weight. The mechanism of weight alteration is not well elucidated but it could be that MO helps substitute for more calorie-dense foods [57]. Fasting blood glucose (FBG) level was normal in non-diabetic groups; it increased significantly in diabetic rats compared to control indicating that experimental diabetic rats truly experienced hyperglycaemia and increased blood glucose has been reported to contribute to the pathogenesis of accelerated cardiovascular disease in diabetes [58]. Following treatment of diabetic rats with MO, FBG level was significantly lowered compared to control and diabetic control. Results showed the anti-hyperglycemic effect of MO regulating high glucose level, demonstrating its ability to decrease blood glucose level [57,59,60] with the presence of terpenoid in MO leaf extract possibly contributing a role.

Glycated haemoglobin level (HbA1c) is a long term indicator of diabetes and diabetic progression is formed when glucose binds to hemoglobin [61-63] which is associated with a poor glycaemic control in diabetic patients [64]. From our study, the HbA1c level was significantly higher in diabetic groups when compared to normal control. Hyperglycemia significantly increased HbA1c in diabetic rats. HbA1c level decreased significantly in diabetic rats only after treatment with MO. Hypoglycemic activity of MO may be due to the presence of α -glucosidase [65]. Our result is consistent with other reports, showing MO's potency to lower glycated haemoglobin level [66] and potentially reduced diabetic complications. Plasma antioxidant status decreased in diabetic rats. Administration of MO to diabetic rats revealed a significant increase in antioxidant capacity which is as a result of the high phenolic contents of MO [56,57].

Free radicals have been implicated in diabetes and its complications resulting in β -cell cytotoxicity [67]. Alteration of antioxidant enzyme activities in a diabetic state is a reflection of the pathogenesis of diabetes [68]. MO increases antioxidant enzyme activities forming a defensive mechanism against free radicals and oxidative stress. Other studies reported similar results [69,70]. An additional contribution to the activity of MO is the presence of chlorogenic acid which

acts natural antioxidant and anti-inflammatory compound [71] and chlorogenic acid has also been shown to reduce glycemic response in rats [21,72].

This study further evaluated SOD, CAT, GPx activities and GSH in the liver and erythrocytes of normal and diabetic rats. In the liver, SOD, CAT activities and GSH decreased significantly in diabetic groups. The decreased actions of SOD, CAT and GSH in diabetic rats may be due to inactivation caused by free radicals. However, MO increased the activities of these enzymes in the diabetic treated group. In non-diabetic rats, SOD activity decreased significantly after treatment with MO. Reduced SOD activity in diabetic rats could be due to a rise in H₂O₂ in affected tissues [18]. Also, CAT activities and GSH decreased in non-treated diabetic group but not significantly. GPx activity was elevated in diabetic rats with a reduction after MO treatment. In an attempt to detoxify hydrogen peroxide produced in a diabetic state, GPx activity increases.

Furthermore, SOD, CAT and GPx activities in erythrocytes were reduced in diabetic rats and increased after MO treatment. GSH concentration increased significantly in diabetic treated groups when compared to non-diabetic groups. Alteration of these antioxidant enzyme activities leads to oxidative stress and injury which was observed in diabetic rats. MO exerts its antioxidant properties by preventing excessive oxidation of macromolecules such as lipids, proteins, carbohydrates and DNA [73]. These antioxidant defence system (SOD, CAT, GPx and GSH) are capable of scavenging and stabilizing free radicals before they attack cells, organs and macromolecules. Chumark et al. [73] demonstrated the antioxidant ability of MO to scavenge free radicals thereby protecting the cells from oxidation.

Functional activities of liver enzymes AST, ALT and ALP which are markers of hepatic damage were also investigated. AST is an important biomarker for the diagnosis of myocardial infarction and amino acid metabolism [74]. We observed a significantly higher activity of AST in the serum of STZ-diabetic induced rats. Administration of MO reduced the activity of this enzyme [75]. Result suggests hepatic damage as shown by elevated AST activity. ALT predominantly found in the liver is a biomarker for hepatocellular injury. Results from the current study showed a significantly

higher ALT activity in diabetic groups when compared to non-diabetic treated rats. The reduction of ALT concentration after MO treatment elucidates the hepatoprotective effect of MO as seen in the study of Fakurazi et al. [35].

Increased activity of ALP is indicative of bile ducts obstruction and a biomarker for hepatic damage. ALP activity in diabetic groups was significantly elevated. Treatment with MO resulted in a significant reduction in ALP activity in diabetic rats compared to diabetic control, confirming previous studies [76,77]. Enzymatic activities of AST, ALT and ALP were elevated in non-treated diabetic group but reduced significantly in MO diabetic treated group. Studies revealed that exposure to STZ increased serum biomarkers associated with hepatic damage. Protective action of MO significantly reduced hepatic enzymes activities.

The liver plays a pivotal role in lipid metabolism. Cholesterol is essential for vitamin D synthesis and hormone metabolism. The normal range is beneficial but when exceeded, it becomes harmful to cellular integrity. Excess cholesterol forms plaque in artery walls, narrowing it and reducing the rate of blood circulation leading to a condition known as atherosclerosis.

In STZ-induced diabetic rats, cholesterol levels were elevated. However, after treatment with MO, cholesterol level was reduced significantly when compared to controls. Also, cholesterol level in non-diabetic treated rats was reduced after MO administration. Our results are in agreement with previous studies which reported hypolipidaemic activity of MO leave extracts, indicating that MO significantly lowered cholesterol level [73]. Manohar et al. [78] also reported similar results in which MO caused a reduction in serum cholesterol level.

Lipoproteins help transfer lipids around the body in the extracellular fluid [79]. LDL is synthesized in the liver by the action of lipolytic enzymes with increased concentrations leading to cholesterol build-up in the arteries [80]. High level of LDL was observed in diabetic rats when compared to non-diabetic rats. Interestingly, after treatment with MO, LDL level in diabetic rats and non-diabetic rats reduced significantly when compared to the control. Other studies have alluded to

the fact that MO has hypoglycemic and hypolipidemic effects which are in tandem with findings from this study [25, 81,82].

HDL mediates the reverse transport of cholesterol from the extra-hepatic tissues to the liver, where other forms of cholesterol are converted to bile acid and excreted [83]. HDL level increased significantly in non-diabetic treated rats compared to non-diabetic rats (positive control). Low HDL levels was seen in diabetic rats which increased after MO treatment when compared to normal control. Reduced HDL levels may result from a decrease in insulin production.

Antioxidant capacity of the liver was measured in diabetic and non-diabetic rats. Treatment with MO led to an increase in liver antioxidant capacity (ORAC) when compared to control groups. ORAC in MO treated diabetic liver increased in comparison to normal control [26].

Chronic Inflammation is associated with diabetes and has been reported to cause severe organ damage in diabetic rats [84-86]. The concentrations of cytokines (IL-6, TNF- α) and chemokine (MCP-1/CCL2) were quantified in the liver homogenate of diabetic and non-diabetic rats in the present study. Cytokines are messenger molecules of the immune system, mediating cellular movement among lymphocytes, macrophages, dendritic cells and other inflammatory cells. Inflammation is one of the markers of oxidative stress. Generation of ROS leads to the upregulation of pro-inflammatory cytokines which have been reported in the diabetic state in rats [87,88]. TNF- α activates endothelial cells attached to corresponding proteins and inflammatory responses. TNF- α has also been implicated in diabetic hepatic disease through diverse biological activities including impairment of insulin secretion and induction of apoptosis and necrosis [89,90].

MO has beneficial effects in various pathological conditions through its anti-oxidative and anti-inflammatory properties. Concentrations of IL-6, TNF- α and MCP-1 increased significantly ($p < 0.05$) in STZ induced rats and a decrease was observed after MO treatment compared to diabetic control. Considering MO's potent anti-inflammatory activity, it can be said to show profound

influence on inflammatory associated diseases and resultant symptoms [91]. On the basis of this study, it can be postulated that MO exerts its hepatoprotective via its anti-inflammatory action.

In this study, non-diabetic treated rats showed no visible lesions when compared to non-diabetic control. Liver specimens from diabetic rats revealed severe hepatocyte necrosis at the centrilobular zone with a large focus of massive haemorrhage into the liver parenchyma, with undefined boundaries when compared to the diabetic treated rats. This may have accounted for the increase in liver weight seen in diabetic rats. After treatment of diabetic rats with MO, liver section showed moderate portal congestion and very mild periportal cellular reaction. MO treatment restored the normal histology of the liver when compared to that of the diabetic positive control group [92]. Furthermore, histological section showed severe hepatocyte necrosis in the liver of the diabetic rats which were cleared in MO treated diabetic rats.

Histology sections of the pancreas of diabetic rat sections showed severe congestion of major interstitial vessels as well as thickening of the interstitial vessel and degenerating islets (1) in comparison to non-diabetic control group. Diabetic rats treated with MO, revealed slightly prominent interstitial connective tissues and rejuvenated islets (2) when compared to diabetic control group. Previous research finding has shown that diabetic pancreatic β -cells have a weak antioxidant defense [63]. The rejuvenated islets in treated diabetic rats could be as a result of MO treatment which is capable of repairing and reversing damage caused by free radicals thereby protecting pancreatic β -cells. Gupta et al. [69] also reported similar results in the pancreatic section after MO treatment.

5.7 Conclusions

From the study, experimental evidence revealed that diabetic rats experienced severe oxidative damage which MO significantly reversed by improving the antioxidant status and by reducing inflammatory mediators via its potent antioxidant and anti-inflammatory properties in a diabetic

state. MO may be able to lower the risk of cardiovascular diseases with its ability to lower the level of cholesterol and low-density lipoprotein but increase high-density lipoproteins.

The study also demonstrated MO's capability to repair damaging effect of hyperglycemia in the liver and pancreatic β -cells with the potential of proffering beneficial effects to individuals with poor glycemic control. It is evident from this study, that MO could be used as a potent antioxidant source.

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Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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CHAPTER SIX

6 Expression of BCL-2 and repression of apoptotic biomarkers in streptozotocin-induced diabetes in male Wistar rats following *Moringa oleifera* administration

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ABSTRACT

Purpose: We investigated the expression of specific apoptotic and anti-inflammatory biomarkers in the liver and kidney of streptozotocin (STZ)-induced diabetes in rats.

Methods: Forty-eight adults rats with weights between 200 and 250 g were randomly divided into four groups; - non-diabetic control (NC), non-diabetic *Moringa* treated (NC + MO), diabetic control (DM) and diabetic *Moringa* treated (DM + MO). Diabetes was induced in Wistar strain rats by a single intraperitoneal injection of STZ (55 mg/kg). Rats were treated with 250 mg/kg of *Moringa oleifera* (MO) extracts. Serum creatinine, albumin, bilirubin level were assessed. Interleukin (IL-1 α), interleukin (IL-12) and interleukin (IL-18) were assayed in the kidney and liver. Kidney and liver sections of rats were assessed for apoptotic cell death markers.

Results: Diabetic rats treated with MO had decreased creatinine and bilirubin levels while albumin significantly increased when compared to non-diabetic control. A significant reduction in interleukin (IL-1 α), interleukin (IL-12) and interleukin (IL-18) was observed in non-diabetic and diabetic treated groups. In the kidney, caspase 3, 9 and NF κ B decreased in diabetic rats treated with MO when compared to normal control as well as untreated diabetic control. There was upregulation of BCL-2 in both the kidney and liver in diabetic treated rats compared to untreated diabetic rats.

Conclusion: This study demonstrates that MO exerted anti-apoptotic anti-inflammatory properties on diabetic rats due to its capacity to reduce nephrotoxic and hepatotoxic damage induced by STZ and to regulate the expression and suppression of specific apoptotic cell death markers.

Key words: *Moringa oleifera*, diabetes, methanol extract, streptozotocin, apoptotic and inflammation.

6.1 Introduction

Diabetes mellitus (DM) is one of the most common autoimmune diseases which cause cellular damage and induces cell death in various organs in diabetic population (Cai et al., 2002; Lee & Pervaiz, 2007). Diabetic nephropathy and hepatopathy are the most common microvascular complications of diabetes causing renal failure and liver damage (Fowler, 2008, Wu & Yan, 2015, WHO, 2016). Diabetes is characterized by hyperglycemia, loss of pancreatic β cells function, expression and repression of specific apoptotic proteins and elevation of inflammatory cytokines coupled with insulin deficiency which is currently becoming a pandemic globally (Roy et al., 2015).

The immune system is structured to provide the defence for combating a number of diseases via several mechanisms. In chronic diabetic state, however, this function deteriorates causing severe damage to vital organs and tissues. DM induced by cytotoxic drugs; streptozotocin is associated with increased generation of reactive oxygen species(ROS) leading to oxidative stress, activation of pathogenic pathways involved in diabetic complication, increased inflammatory mediators and the production of pro-apoptotic factors (Park et al., 2001; Chawla et al., 2016).

Apoptosis plays an important role in the developmental processes as well as acting as a defence mechanism and regulating the immune system (Elmore, 2007; Norbury & Hickson, 2001). Apoptosis programs damaged cells for self-destruction in a controlled process. It is executed by caspases which are critical for maintaining tissue homeostasis that involves the prompt removal of unwanted cells for maintenance of specific organs (Nakanishi et al., 2009). Defects in apoptosis regulatory mechanism are implicated in diabetic states and may contribute to the pathogenesis of a wide range of diseases while excess apoptosis remains an underlying cause for cell loss in various disease states including diabetes (Matés & Sánchez-Jiménez, 2000).

Caspase 3 is an executioner caspase, highly expressed in diabetic individuals where the neural cells are lost with increased excessive cell death regulated by B-cell lymphoma 2 (BCL-2) (Korsmeyer, 1999; Hui et al., 2004; Bruey et al., 2007). BCL-2 is an anti-apoptotic protein that regulates cell death and promotes cell survival as well as to determine the commitment of cells to apoptosis (Ahmad et al., 2009; Wang, 2015).

Chronic inflammation is associated with diabetes and has been reported to cause severe organ damage in diabetic rats (Mohamed et al., 2016). Significant levels of inflammatory cytokines have been reported in diabetic animal models (Ayepola et al., 2014; Liang et al., 2015; López-López et al., 2016). MO and other medicinal plants have been found to be effective in traditional medicine as a potent anti-inflammatory agent with little or no adverse effect (Ezeilo & Green 1977).

Although many drugs have been designed for the treatment and management of diabetes, there are still limitations in the use of anti-hyperglycemic medications due to known side effects, high cost, limited action and secondary failure rates. Despite improvements in the treatment and management of diabetes, the rate seems to be accelerating drastically. *Moringa oleifera* (MO) tree is distributed widely in the tropics and subtropics and has been reported to possess antidiabetic, antioxidant and other medicinal properties which may be helpful in managing diabetes and its associated complications (Stohs & Hartman, 2015; Gopalakrishnan et al., 2016; Farooq et al., 2012).

This study investigated the anti-apoptotic and anti-inflammatory properties of methanol leaf extract of MO in the liver and kidney of diabetic and non-diabetic animal model. The mechanism of diabetes linked to apoptosis is not clearly known but our aim is to elucidate the fact that the expressions of certain apoptotic and inflammatory markers are upregulated in the diabetic state.

6.2 Materials and Methods

6.2.1 Preparation of *Moringa oleifera* extract

MO leaves were harvested from Forestry Research Institute of Nigeria (FRIN), identified and authenticated at FRIN and a specimen deposited in the herbarium with specimen number FHI-110287. The leaves were dried under shade and blended to powder. One kilogram (1 kg) powder was extracted with n-hexane for 24 hr to defat and re-extracted with methanol. The filtrate was dried under reduced pressure at 40°C in a rotatory evaporator and stored until use.

6.2.2 Animal care and feeding

6.2.2.1 Diabetes Induction

A single dose of diabetogenic agent streptozotocin (STZ), 55 mg/kg (Sigma Chemical Co, St. Louis, USA) was used to induce diabetes in rats. The drug was dissolved in freshly prepared ice-cold 0.1 M citrate buffer (pH 4.5), thereafter injected intraperitoneally (Jaiswal et al., 2013). Only rats with an elevated fasting blood glucose level of ≥ 18 mmol/L three days post-STZ injection were included in the diabetic group before the commencement of treatment. The administration of MO to rats only commenced on the fourth day after rats were confirmed to be diabetic and this was considered as the first day of treatment.

6.2.2.2 Experimental design

Forty-eight healthy male Wistar rats weighing 200-250 g were obtained from Charles River (Margate, UK) for the study. Rats were housed in stainless steel cages under routine laboratory conditions at the Stellenbosch University Animal Research Facility (Stellenbosch University, Tygerberg, CA, South Africa). They received water, standard rat chow (SRC, Aquanuro, Malmesbury, South Africa) and proper care *ad libitum*. Twenty four normal rats and twenty-four diabetic rats were randomly divided into four experimental groups.

The rats were randomly assigned into four (4) groups of twelve rats (n = 12). Group 1: (Normal control) rats were non-diabetic administered with the vehicle and standard rat chow (SRC) only. Group 2: (Normal + *Moringa* treated) rats were non-diabetic treated with MO extract

(250 mg/kg) and SRC. Group 3: (Diabetic control) rats were diabetic administered with the vehicle and SRC without receiving the plant extract. Group 4: (Diabetic + *Moringa* treated) rats were diabetic treated *Moringa oleifera* extract (250 mg/kg) and SRC. Based on preliminary investigations and following Kar et al. (2003), Efiang et al. (2013) and Tabassum et al. (2013), a dose of 250 mg/kg MO extract was the most suitable and this was used for our study. MO extracts were dissolved in distilled water and orally administered daily for six (6) weeks.

6.2.2.3. Serum and tissue preparation

After the last treatment, rats were fasted overnight and anaesthetized with sodium pentobarbital injection (60 mg/kg) for painless and rapid death. Blood was collected from rats via rat's abdominal aorta into a serum clot activator tubes and centrifuged at 4,000 g for 10 minutes at 4 °C to obtain serum then stored at -80 °C. The kidney and liver were excised, rinsed in saline phosphate-buffer, chopped in small pieces and homogenized. Homogenate was centrifuged at 4 °C at 15000 rpm for 10 minutes and stored at -80 °C prior to analysis

6.2.3 Analysis

6.2.3.1 Determination of serum creatinine, albumin and bilirubin concentrations

Creatinine, albumin and bilirubin concentrations were measured in the serum using Randox kits on the automated Randox Daytona from Randox Laboratories Limited kit, Crumlin, United Kingdom. All protocols were followed according to the manufacturer's manual.

6.2.3.2 Inflammatory markers in the kidney and liver

The inflammatory cytokines interleukin-1 α (IL-1 α), interleukin-12 (IL-12) and interleukin-18 (IL-18) were assayed in the kidney and liver supernatant using Luminex MILLIPLEX[®] MAP rat cytokine magnetic beads kits (Merck Millipore, Billerica, MA, USA). The procedure followed was in accordance with the manufacturers' manual (Merck Millipore). Results were read with the Bio-plex[®] platform (Bio-Rad Laboratories, Hercules, CA, USA) and the Bio-Plex Manager[™] software 6.0 was used for the acquisition of beads and median fluorescent intensity analysis. Results were given in pictogram per milligram cell protein.

6.2.3.3 Immunohistochemistry

Paraffin embedded kidney and liver blocks were selected from all experimental groups, sectioned to 3 µm using a Leica RM2125 RTS microtome (Leica Microsystems, Inc., Buffalo Grove, IL, United States) and placed on charged slides. The paraffin sections were deparaffinized and rehydrated in xylene and graded ethanol, respectively. The sections were incubated at 60°C for 1 hour followed by pretreatment with heat-induced epitope retrieval (HIER, EDTA pH 8) at 98°C for 20 minutes to expose antigen sites allowing antibodies to bind. Specific antibodies obtained from Abcam anti-caspase 3 (ab4051), anti-caspase 9 (ab52298), anti-BCL-2 (B-cell lymphoma 2) (59348), anti-NFKβ (nuclear factor kappa β) (ab7971) and anti-p53 (ab131442) were applied to kidney and liver slides. Each antibody was diluted 1:100 with bond primary antibody diluent (Leica Biosystems, Newcastle, United Kingdom).

Staining was carried out using the Leica automatic stainer XL (Leica Microsystems GmbH, Wetzlar, Germany) then placed in a Bond Max Automated Immunohistochemistry Vision Biosystems (Leica Microsystems GmbH, Wetzlar, Germany). Peroxidase blocking was done for 5 minutes using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH).

The tissues were incubated and washed with primary antibody for 15 minutes. The wash process was repeated with post primary antibody. The tissues were then incubated with polymer for 8 minutes and 3,3'-diaminobenzidine tetrahydrochloride (DAB) Chromogen solution was mixed for 10 minutes to enhance the product of the reaction yielding a brown stable end-product. Mayer's hematoxylin was applied for 5 minutes and tissue slides were thereafter rehydrated manually with graded alcohol then xylene. Slides were mounted with DPX (dibutyl phthalate xylene) mountant from Ace Associated Chemical Enterprises (Johannesburg, South Africa) and all slides were viewed under a light microscope.

Slides were observed based on the colour intensity of the immunohistochemical staining, a positive result was indicated by the presence of a dark-brown nuclear stain. The slides were

read using ImageJ Immuno Profiler software by an immunopathologist who was blind to the study. The positive intensities were quantified statistically and represented graphically.

6.3 Statistics

Immunohistochemically stained images were quantified using ImageJ Immuno Profiler software (Version 10.2 image analysis). Positive intensities are represented graphically as mean \pm standard deviation. Other analysis were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons for post hoc test (GraphPad Prism version 7.00, La Jolla, CA, USA). Values are represented as mean \pm SD and considered significant with p value < 0.05 .

6.4 Results

6.4.1 Effect of MO on serum creatinine, albumin and bilirubin concentrations

Assessment of serum creatinine, albumin and bilirubin concentrations is shown in table 16. Creatinine level in diabetic control rats was higher than in normal control group. Subsequent treatment with MO decreased creatinine level in diabetic and non-diabetic rats compared to normal control. A significantly low albumin level was observed in diabetic rats compared to normal control while treatment with MO showed an increased level when compared to diabetic control. Total Bilirubin concentration in diabetic control rats significantly ($p<0.05$) increased and following MO administration this concentration significantly decreased when compared to diabetic control. In response to apoptotic markers levels of creatinine, albumin and bilirubin were increased.

Table 16: Serum creatinine, albumin and bilirubin concentrations

Serum	Groups			
	NC	NC+MO	DM	DM+MO
Creatinine (g/L)	50.21 \pm 0.81	49.31 \pm 3.01	52.23 \pm 2.05	51.06 \pm 1.00
Albumin (g/L)	32.50 \pm 0.89	39.36 \pm 3.03 ^{ab}	27.55 \pm 1.17 ^a	29.69 \pm 0.94 ^a
Total bilirubin (g/L)	2.45 \pm 0.33	1.76 \pm 0.49 ^b	5.34 \pm 1.18 ^a	2.74 \pm 0.60 ^b

All values are presented as mean \pm standard deviation. Where ^a $p < 0.05$ indicates that values are significant compared to non-diabetic control (NC), ^b $p < 0.05$ indicates that values are significant compared to diabetic control (DM). Groups: NC; Normal control, MO; normal control + *Moringa oleifera* treated, DM; Diabetic control, DM + MO; diabetic + *Moringa oleifera*-treated.

6.4.2 Effect of *Moringa oleifera* on inflammatory cytokines in the kidney and liver

Table 17 shows the anti-inflammatory properties of MO on the kidney and liver inflammatory cytokines of diabetic and non-diabetic rats. We studied three inflammatory cytokines IL-1 α , IL-12 and IL-18 in the liver and kidney. In the kidney and liver, the concentration of IL-1 α , IL-12 and IL-18 increased significantly ($p < 0.05$) in diabetic groups when compared to normal control. However, after administration of MO to diabetic-treated groups, cytokines concentrations were reduced in the liver (IL-1, IL-18) compared to diabetic control. IL-18 was the only cytokine that reduced in the kidney of diabetic treated rats when compared with diabetic control.

Table 17: Inflammatory cytokines in the kidney and liver

Groups	kidney			Liver		
	IL-1 α (pg/ml)	IL-12 (pg/ml)	IL-18 (pg/ml)	IL-1 α (pg/ml)	IL-12 (pg/ml)	IL-18 (pg/ml)
NC	227.6 \pm 9.79	791.50 \pm 30.27	1970.00 \pm 82.73	1390.00 \pm 51.17	631.70 \pm 37.51	7087.00 \pm 454.00
NC+MO	217.80 \pm 20.70 ^b	751.40 \pm 44.12 ^b	1683.00 \pm 138.70 ^{ab}	1320.00 \pm 49.77 ^b	588.30 \pm 63.43 ^b	5691.00 \pm 446.30 ^{ab}
DM	297.00 \pm 14.98 ^a	1401.00 \pm 54.18 ^a	2248.00 \pm 123.20 ^b	2025.00 \pm 63.81 ^a	1071.00 \pm 52.55 ^a	7587.00 \pm 244.00
DM+MO	283.80 \pm 15.11 ^a	1360.00 \pm 76.78 ^a	1125.00 \pm 82.62 ^{ab}	1257.00 \pm 35.81 ^{ab}	1051.00 \pm 54.04 ^a	5926.00 \pm 525.40 ^{ab}

All values are presented as mean \pm standard deviation. Where ^a $p < 0.05$ indicates that values are significant compared to non-diabetic control (NC), ^b $p < 0.05$ indicates that values are significant compared to diabetic control (DM). Groups: NC; Normal control, MO; normal control + *Moringa oleifera* treated, DM; Diabetic control, DM + MO; diabetic + *Moringa oleifera*-treated.

6.4.3 Effect of *Moringa oleifera* on apoptotic cell death markers on the kidney

Figure 21 illustrates immunohistochemical staining intensity of apoptotic cell death markers: caspase 3, caspase 9, BCL-2, NFK β p105 and p53 in the kidney of diabetic and non-diabetic rats. The expression of cell death marker is indicated by a brownish nuclear stain of different intensities. The positive intensity of stain was quantified graphically and pictorially. A significant ($p < 0.05$) increase in the expression of caspase 3, caspase 9, NFK β in diabetic rats was observed when compared to normal control, while the intensity of p53 also increased relative to normal control but not significantly ($p > 0.05$). The expression of BCL-2 was unchanged in diabetic control rats compared to normal control. Treatment with MO, however, reduced expression of caspase 3, caspase 9, NFK β , p53 and an increased expression of BCL-2 relative to the control and diabetic groups.

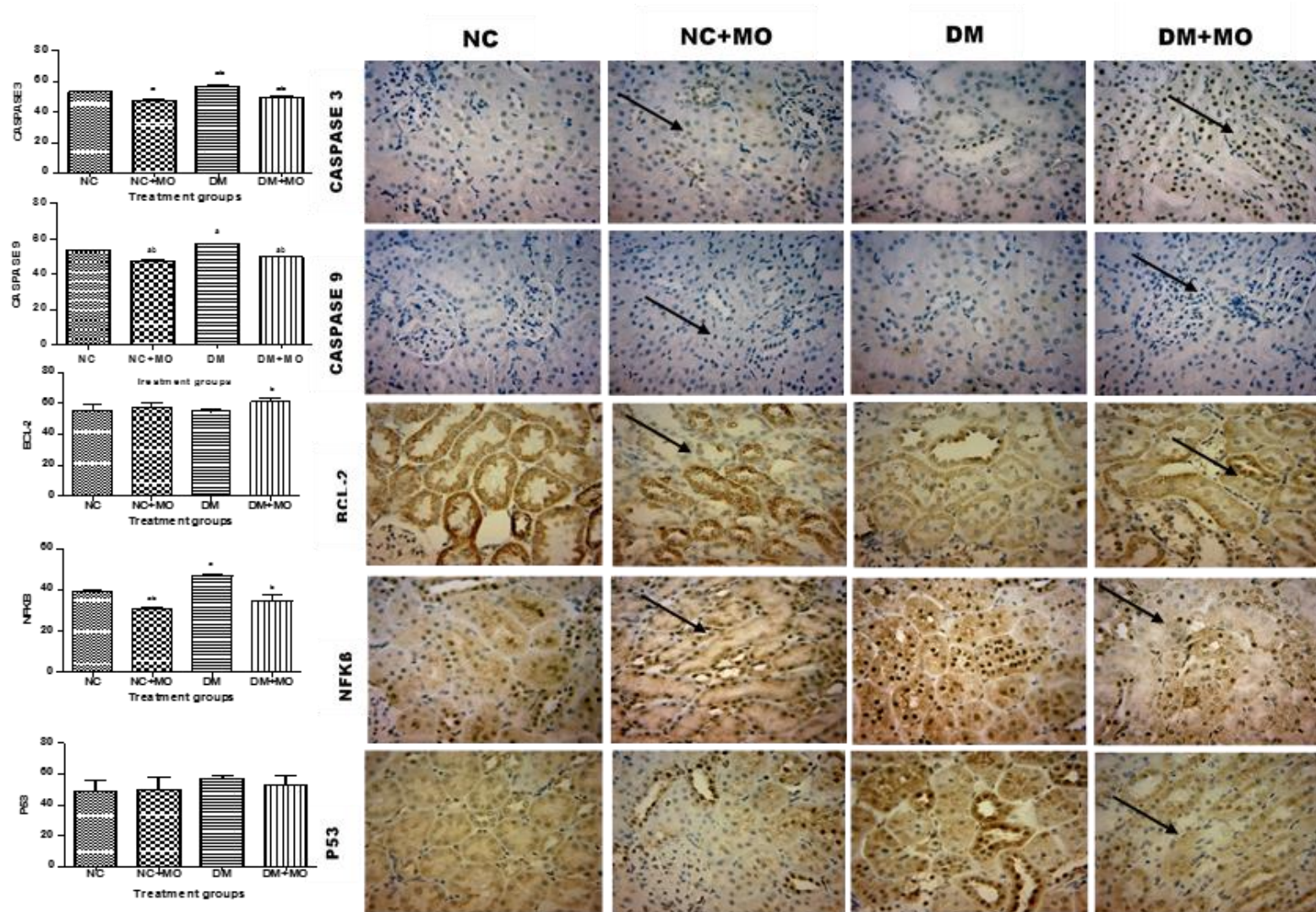


Figure 21: Immunohistochemical representation of apoptotic cell death markers of the kidney tissue labelling with anti-caspase 3, anti-caspase 9, anti-BCL-2, anti-NFK β p105 and anti-p53 at 1/100 dilution and X400 magnification. Varying depth of the expression is indicated by dark-brown staining. Graph bars are presented as mean \pm standard deviation: ^a $p < 0.05$ indicating values are significant compared to non-diabetic control (NC), ^b $p < 0.05$ indicating values are significant compared to diabetic control (DM). Groups: NC; Normal control, MO; *Moringa oleifera*-treated control rats, DM; Diabetic rats, DM + MO; *Moringa oleifera*-treated diabetic rats.

6.4.4 Effect of *Moringa oleifera* on apoptotic cell death markers on the liver tissues

Figure 22 illustrates the effect of MO on expression apoptotic markers: caspase 3, caspase 9, BCL-2, NFK β p105 and p53 in the liver of diabetic and non-diabetic rats. The expression of apoptotic cell death marker is indicated by a brownish nuclear stain of different intensities. The positive intensity was quantified graphically and pictorially. Significant ($p < 0.05$) increase in intensities of caspase 3, caspase 9, NFK β relative to normal control was observed. The intensity of p53 also increased relative to normal control but not significant ($p < 0.05$). The intensity of BCL-2 increased in diabetic treated rats when compared to normal control. Treatment with MO resulted in a decreased expression of caspase 3, caspase 9, NFK β , p53 and an increased expression of BCL-2 relative to the control and diabetic groups.

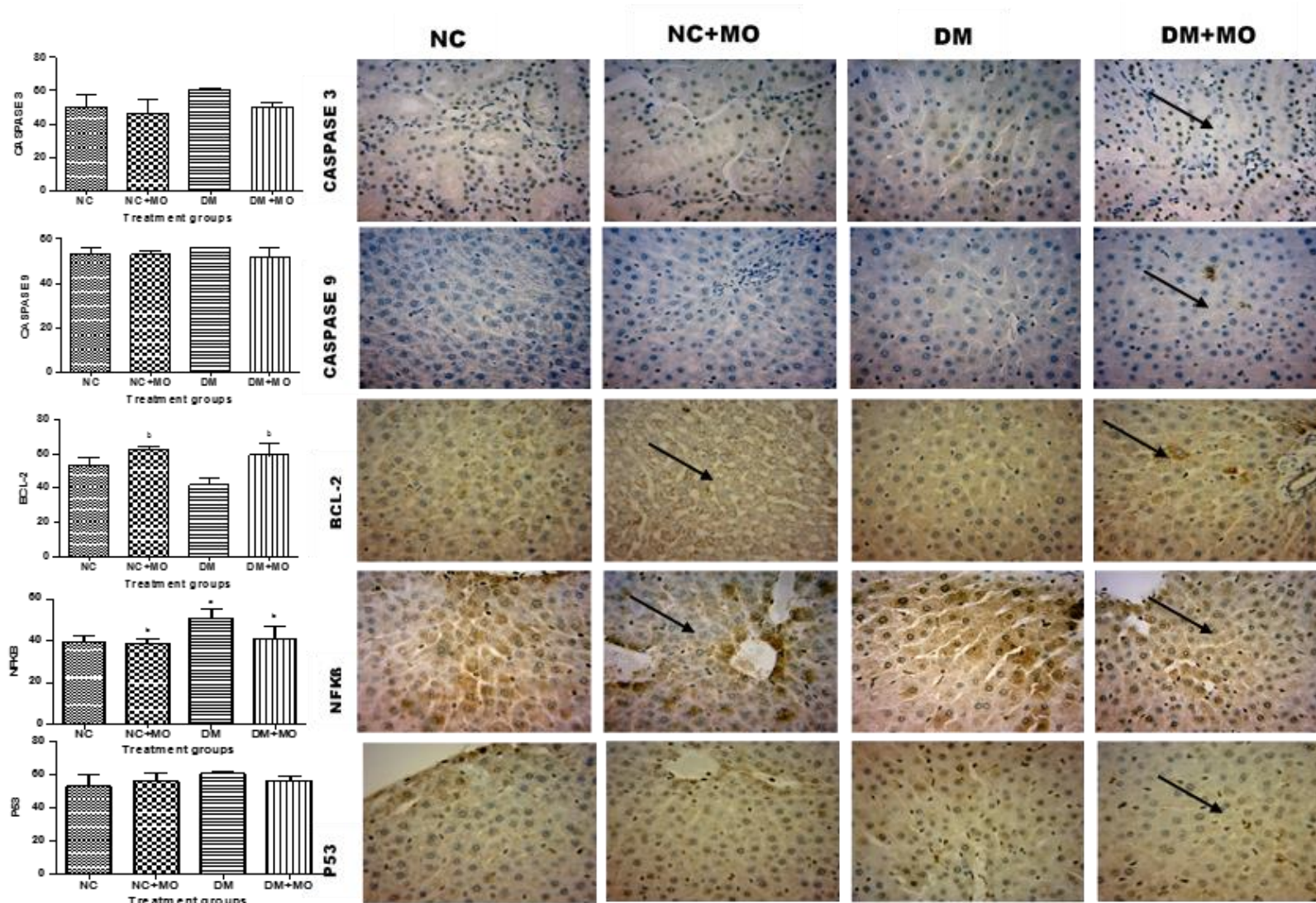


Figure 22: Immunohistochemical representation of apoptotic cell death markers of the liver tissue labelling with anti-caspase 3, anti-caspase 9, anti-BCL-2, anti-NFKB p105 and anti-p53 at 1/100 dilution and X400 magnification. Varying depth of the expression is indicated by dark-brown staining. Graph bars are presented as mean \pm standard deviation: ^a $p < 0.05$ indicating values are significant compared to non-diabetic control (NC), ^b $p < 0.05$ indicating values are significant compared to diabetic control (DM). Groups: NC; Normal control, MO; *Moringa oleifera*-treated control rats, DM; Diabetic rats, DM + MO; *Moringa oleifera*-treated diabetic rats

6.5 Discussion

Damage to β -cells of the pancreas resulted in increased glucose levels and the release of reactive oxygen species (ROS) (Altan et al., 2007; Dogan et al., 2015). Diabetic rats showed upregulation and downregulation of certain inflammatory and apoptotic markers and a cascade of ROS generation in the liver and kidney of rats. These ROS activate the release of superoxide radicals that cause damaging effects and oxidize biological molecules at the cellular level forming bioactive oxidative products (Rytter, 2009).

Creatinine (CR) is a product of muscle breakdown from creatine phosphate to creatinine catalyzed by creatine kinase (Li et al., 2016). The kidney functions in maintaining normal blood CR level by filtering CR from the blood by renal glomerular filtration and excreting it via urine (Ferguson & Waikar, 2012). However, damage to the kidney will increase the level of creatinine synthesis and elevated CR level in the blood, thus indicating abnormal kidney function (Wyss & Kaddurah-Daouk, 2000). In this study, serum creatinine level was elevated in the diabetic groups presumably due to poor creatinine clearance resulting from STZ-induced diabetes. However, treatment with MO showed no change in CR when compared to non-diabetic normal control. Results are in conformity with previous studies (Al-Malki & El Rabey, 2015; Gao et al., 2016).

Albumin is a metal binding protein synthesized in the liver, functioning to stabilize blood volume and regulate vascular fluid exchange (Bhagavan & Chung-Eun, Ha, 2015). Abnormal serum albumin level is indicative of kidney or liver damage (Bhat et al., 2016). Serum albumin test was performed in this study, and hypoalbuminemia was seen in diabetic rats when compared to non-diabetic control groups in this study. This suggests adrenocortical stimulation giving rise to the breakdown of albumin and sodium retention (Bhagavan & Chung-Eun, Ha, 2015). Also, this indicates the initiation and progression of diabetic nephropathy (Elsherbiny & Al-Gayyar, 2016; Al-Malki & El Rabey 2015).

Bilirubin, a non-enzymatic endogenous antioxidant is an end product of hemoglobin and ineffective erythropoiesis (production of red blood cells) (Dekker et al., 2011; Murray et al., 2012). Serum total bilirubin (T-bill) is an assessment of both conjugated and non-conjugated bilirubin and acts as a marker of liver damage and can be used to diagnose any blockage to the bile duct. The current study observed a significantly increased level of T-bilirubin in diabetic groups relative to normal control rats; this level was seen to reduce greatly after MO administration. The elevated level of T-bill in of STZ rats may be in an attempt to suppress the generation of ROS to protect cells from damage or may be indicative of haemolytic anaemia (Dekker et al., 2011). Increased ROS levels are associated with hyperbilirubinaemia which is grossly dependent on the rate of red cell destruction and excretion of bilirubin (Mohan & Chandrakumar, 2015).

In diabetic individuals, creatinine, albumin and bilirubin levels are assessed to ascertain the normal function of the kidney and liver which in this study showed alterations. Administration of MO reduced only albumin significantly in diabetic treated rats preventing the depletion of liver proteins and showing its nephroprotective and hepatoprotective activity. These may be as a result of carotenoids and phenolics content of MO (Soliman, 2013; Olatosin et al., 2014). Our results agree with previously reported studies on the protective effects of MO on serum biochemical parameters (Adedapo et al., 2009; Olayemi et al., 2016; Kim et al., 2016).

Pro-Inflammatory cytokines are signalling intracellular polypeptides. Inflammatory markers in the kidney and liver of non-diabetic and diabetic rats were determined. Inflammatory responses are activated by several physiological and metabolic factors which are involved in the onset and progression of diabetic nephropathy and diabetic hepatopathy (Joly et al., 2015; Elsherbiny & Al-Gayyar, 2016).

Concentrations of IL-1 α , IL-12 and IL-18 were significantly high in the kidney and liver of diabetic groups due to hyperglycemia when compared to normal control. Chronic inflammation was seen in tissues of diabetic rats in response to injury characterized by migration of

leucocytes, vasodilation of local blood vessels and increased cytokines level (Trinity et al., 2016; Al-Harbi et al., 2016). High concentration of inflammatory cytokines in the islet microenvironment led to induction of apoptotic signalling cascade (Manna et al., 2010). However, elevated concentration of these cytokines decreased considerably after MO administration. Results from this present study indicated that inflammatory markers were upregulated in the kidney and liver of diabetic rats in response to injury caused by oxidative stress. This study is in consonance with findings from other studies that observed MO's potency when compared to standard anti-inflammatory agents (hydrocortisone and indomethacin) (Georgewill et al., 2010; Waterman et al., 2014).

Immunohistochemistry (IHC) is widely used in the assessment and diagnosis of abnormal cells and the progression and treatment of cancerous tumours. These diagnostic techniques have been used in animal studies as well as clinical studies (Oikarinen et al., 2010; Larson et al., 2015; Ahmad et al., 2016). For this study, IHC was used in the assessment of apoptotic cell death markers in the kidney and liver of diabetic rats. The expression and repression of caspase 3, caspase 9, BCL-2, NF κ B p105 and p53 protein were performed in the kidney and liver of diabetic and non-diabetic rats. The induction of diabetes with STZ in rats resulted in hyperglycemia which activated the high rate of synthesis of inflammatory proteins, generation of ROS and activation of apoptotic response (Ghosh et al., 2015; Gopalakrishnan et al., 2016).

Apoptosis a programmed cell death is triggered by several factors including damage to DNA, expression of a disease and receptor mediated signal (Elmore, 2007). BCL-2 is a protooncogene regulatory protein, located in the outer membrane of the mitochondria, nuclear envelope and in the endoplasmic reticulum. It determines the commitment of cells to apoptosis. BCL-2 activates caspase 9 in the mitochondria which then activates pro-caspase 3 then caspase 3 leading to the degradation of DNA and other substrates. Caspase 9 which is known as an activator caspase initiates apoptosis. Caspase 3, a pro-apoptotic caspase of the family of cysteine proteases, is known as an executioner caspase and plays a very crucial role in the execution of apoptosis (Pan et al., 2012).

BCL-2 promotes cell survival, protects the cell from ROS damage and inhibits pro-apoptotic proteins (Bax and Bak) whose function is to activate cell death (Youle & Strasser, 2008). The inhibition of pro-apoptotic proteins prevents the release of cytochrome C, deoxyadenosine triphosphate (dATP) and apoptotic protease activating factor-1 (APAF-1) which are important signals in the apoptotic cascade (Barclay et al., 2015). Once this happens, caspase 9 known as an activator caspase, will not be expressed and caspase 9 will not be activated. To infer from this study, the BCL-2 protein was not expressed in the kidney and liver of diabetic rats, owing to the presence of ROS which activated the release of pro-apoptotic proteins and cytochrome C, dATP, APAF-1 thereby facilitating the activation and expression of caspase 9 and caspase 3 (Cain, 2002). Treatment with MO increased BCL-2 expression in both kidney and liver tissues. It is likely that low expression of BCL-2 may be as a result of diagnosis of some dead cells in the tissues with the increased expression of caspase-3 (Glantz et al., 2006).

The expression of caspase 3 in the kidney and liver tissues of diabetic rats exhibiting the highest intensity where the neural cells were lost showed increased excessive cell death, compared to the control and other groups. This may be consequent to cell damage which is a major cause of cell death. Similar studies have been reported in animal models (Manna et al., 2010; Peixoto et al., 2015). The improvement observed after treatment with MO may be a resultant effect of the anti-apoptotic and anti-proliferative activity of MO which reduced the expressions of these proteins. MO leaves have been reported to contain anti-cancer constituents such as benzyl isothiocyanate, glucosinolates and niazimicin (Hermawan et al., 2012).

NFK β is a transcription factor that regulates programmed cell death; it serves as a switch for turning on certain immune and inflammatory responses (Arumanayagam & Arunmani, 2015). P53 is a tumour suppressor gene involved in cellular response to DNA damage (Marcel et al., 2015). It arrests cells in the G-phase allowing repairs before entry into the S-phase, on account of severe damage. C-Jun N-terminal kinases (JNKs) is activated by stress signals which

activate the expression of p53 and NFK β after DNA damage. This is indicated in our study by brown stained cell nuclei in diabetic groups which are markedly pronounced. NFK β and P53 were expressed in the diabetic group, although expression of NFK β was higher showing the pathogenesis of diabetic nephropathy and hepatopathy (Wang et al., 2012). The level of expressions of these apoptotic markers decreased after treatment with MO. MO suppressed NFK β and p53 activities which were increased as a result of damaging effects of ROS. This presumably owes to MO enhancing cellular antioxidant defence and minimizing abnormal cell proliferation. Results are consistent with findings from other studies (Elsherbiny & Al-Gayyar, 2016; Peixoto et al., 2015; Hamza, 2010).

6.6 Conclusion

In conclusion, this study compared inflammatory and apoptotic markers in the kidney and liver of diabetic and non-diabetic rats. The study demonstrates the anti-inflammatory and anti-apoptotic properties of MO showing its potential to reduce renal and hepatic damage induced by STZ and to regulate the expression and suppression of selected apoptotic cell death markers. Its constituents can act as a natural anti-inflammatory and anti-cancer source considering its effectiveness in reversing some of the alterations caused by STZ.

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Ethical issues

This study was approved by the Research Ethics Committee of Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences, South Africa with ethic no-CPUT/HW-REC 2014/AO8. All protocol for animal handling were according to the principles of laboratory animal care of the National Institutes of Health Guide for the care and use of laboratory animals

of the National Academy of Science (NAS) and published by the National Institutes of Health (publication no. 80-23, revised 1978).

Conflict of Interest

There is no conflict of interest to be declared in this study.

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CHAPTER SEVEN

7 General Discussion

DM is an endocrine disease of multiple aetiologies associated with the absence or deficiency of insulin secretion characterized by hyperglycemia with changes in carbohydrate, lipid and protein metabolism. In this study, hyperglycemia was induced with a single dose of freshly prepared streptozotocin (STZ) (55 mg/kg) to overnight fasted rats. The mechanism of action of STZ in experimental animals led to the destruction of beta cells of the pancreatic islet (Akbarzadeh, et al., 2007; Al-malki et al., 2015). Consequently, STZ toxicity causes modifications to cell function, ATP dephosphorylation reaction and helps xanthine oxidase generate ROS and superoxide radical as well as hydrogen peroxide (Wright et al., 2006; Ayepola et al., 2013) causing damaging effects on various organs such as the liver, kidney and pancreas.

Conventional drugs used to treat diabetes are known to have severe side effects, not easily accessible and not affordable by the majority of people in developing countries. This study elucidates the potential effects of *Moringa oleifera* (MO) leaf extract to manage, treat diabetes and the associated complication in male Wistar rats. The plant possesses antioxidant and medicinal properties that are believed to be helpful in the treatment of diabetes.

Forty-eight (48) adult male Wistar strain rats were randomly divided into four 4 groups with 12 rats per group: Normal control (NC), *Moringa oleifera* treated control rats (MO), Diabetic rats (DM), *Moringa oleifera* treated diabetic rats (DM+MO). MO and DM +MO groups received *Moringa oleifera* methanol extract at a dose of 250 mg/kg/b.wt. via oral gavage for six (6) weeks. Diabetic rats with glucose level ≥ 18 mol/L were included into diabetic groups for the experimental work.

At the end of the treatment, all rats were sacrificed using sodium pentobarbital to ensure unconsciousness of rats while death occurred ensuring rapid and painless death. Blood samples were obtained via the rat's abdominal aorta into a lithium heparin plasma separator tubes and

serum clot activator tubes. The whole kidney, liver and pancreas were quickly excised from each rat, washed in ice-cold phosphate-buffered saline, blotted, weighed and frozen in liquid nitrogen after which they were prepared for biochemical analysis.

In vivo study showed the resulting effect of diabetes induction using STZ. Hyperglycemia was successfully induced in the rats which were confirmed in the blood, kidney and liver biochemical markers. Figure 23 is a schematic diagram of the entire experimental procedures and implications of diabetes in the rats are indicated by red arrows, blue arrows indicate either an increase or decrease in specific biomarkers.

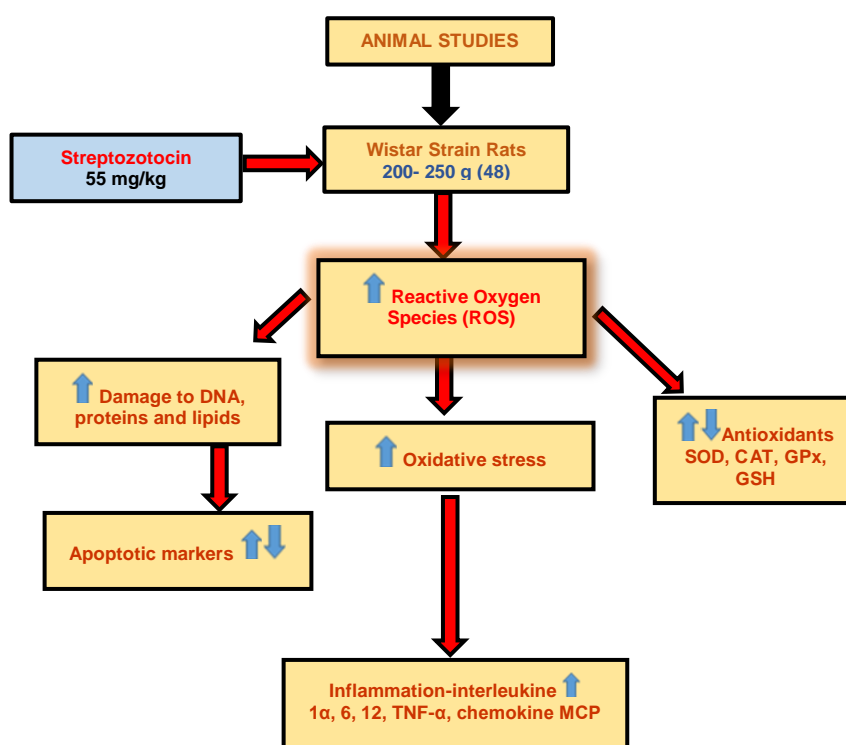


Figure 23: Schematic diagram of the entire experimental procedures and implications of STZ-induced diabetes in rats are indicated by red arrows showing increase or decrease in the biomarkers determined

In vitro study was performed on the aqueous, methanol and petroleum ether extract of MO. Figure 24 shows an overview of the experiment carried out on MO extract. The results are presented in five different chapters focusing on the modulatory effects of the methanol extract of MO in the assessment of its anti-hyperglycaemic, anti-inflammatory, antioxidant and apoptotic activities in diabetes-induced male Wistar rats.

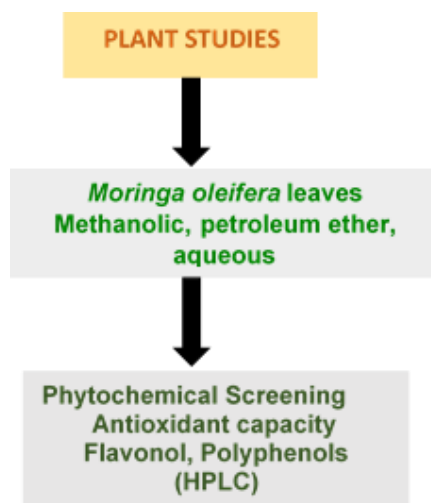


Figure 24: Overview of the phytochemical screening evaluated in *Moringa oleifera* leaves

In this study, the antioxidant and plant chemicals of MO were quantified, identifying the protective and disease preventing activities present in aqueous, methanol and petroleum ether extract of MO leaves. Estimation of oxygen radical absorbance capacity (ORAC), trolox equivalence antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), total polyphenol, flavonoids and flavonol content of aqueous, methanol and petroleum ether extract of MO leaves were quantitatively determined. Specific polyphenols and flavonols content were identified in the methanol extract of MO using HPLC (Agilent Technology1200 series). Antioxidant capacity was used to measure the antioxidant potency of the leaf extract elucidating its free radical scavenging ability. Results indicated a very high radical scavenging capability of MO.

MO showed high phytochemical constituents, particularly demonstrating a high level of total polyphenol, flavonol and flavonoid. The study deduces that MO is capable of preventing or reducing the oxidation of other molecules generally by trapping free radicals and reducing the development of inflammatory cytokines due to its high phenolic contents (Verma et al., 2009). MO also functions by donating a hydrogen atom to lipid radical, quenching singlet oxygen and removing molecular oxygen (Alhakmani et al., 2013). The phytochemical content is responsible for the antidiabetic and antioxidant properties of MO relating more specifically to its total polyphenols, total alkaloids and flavonols and antioxidants identified in the leaves of the plant.

In vivo experiments were carried out on the kidneys, liver, pancreas and blood fractions and in vitro study was performed on the extract used for the experiment. Schematic diagram (Figure 25) shows an overview of the modulatory effects of MO in rats exposed to STZ, the green arrows signify the increase or decrease in the concentration of specific biomarkers.

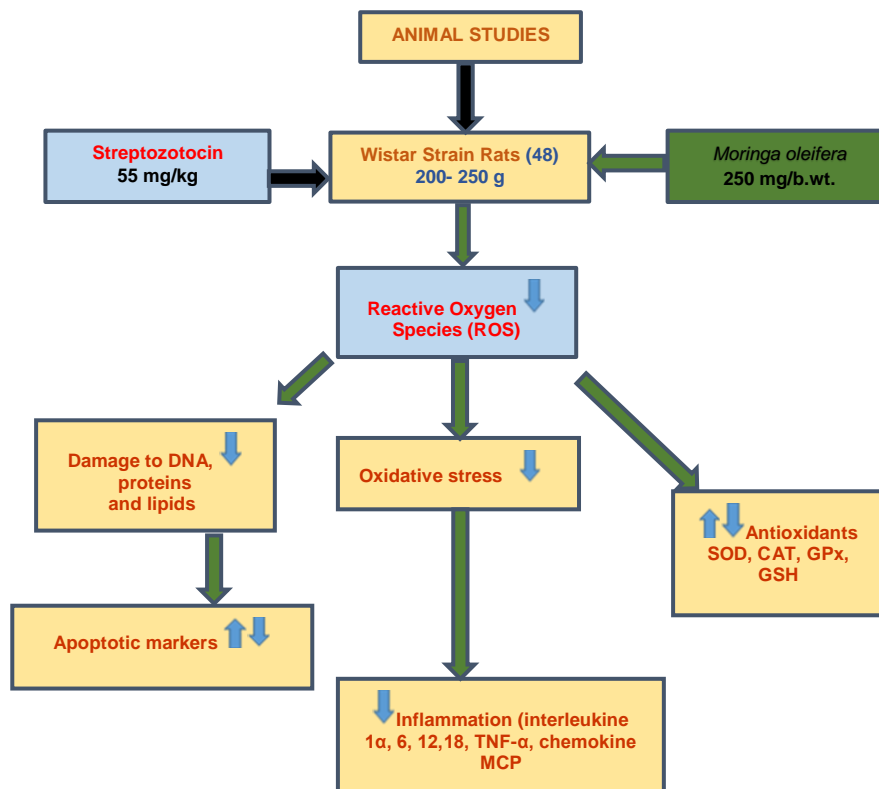


Figure 25: Schematic diagram shows an overview of the modulatory effect of MO in rats exposed to STZ. Green arrows signify the increase or decrease in biomarkers

We also observed changes in the size of organs. Increased kidney and liver size was seen while a decreased pancreas size was observed in diabetic rats compared to the non-diabetic controls. The increased size is a sign of acute inflammation due to hyperplasia (rapid production of the cell leading to enlarged tissue) and hypertrophy (enlargement of cell components) of the kidney (Navarro- González & Mora-Fernández, 2008; Thomas et al., 2008). Liver of diabetic rats increased, suggesting hepatomegaly as a result of the enlargement of tubular cell lining and fatty infiltration (Al-Qattan et al., 2016). Reduction in body weight of diabetic rats was observed consequent to hyperglycemia (Fang et al., 2002). Persistent hyperglycemia may lead to the production of free radicals which attack insulin-producing cells (beta cells) leading to glucose deprivation, promoting protein breakdown (weight loss) in order to generate energy (Jaiswal et al., 2009).

Insulin is produced by β -cells of the pancreas in response to increased glucose levels in the blood and is responsible for the reduction in blood glucose levels (Toma et al., 2012). Elevated plasma, serum and fasting blood glucose levels were observed in diabetic rats. Excess glucose enters the mitochondrial releasing ROS, consequently destroying pancreatic β -cells. A decreased glucose level was seen in MO treated groups compared to controls. This implies that MO is able to increase the ability of insulin to lower glucose by stimulating pancreatic β -cells to produce insulin. This suggests a potential hypoglycaemic of MO and our result is in consonance with findings of Yassa and Tommy (2014). STZ-induced diabetes caused an increase in glycated haemoglobin level in hyperglycemic rats, however, treatment with MO reduced this levels. The result is consistent with other reports, showing MO's potency to lower glycated haemoglobin level indicating a good glycaemic control (Umar et al., 2011; Veeranan et al., 2011).

Serum biochemical markers such as total protein, creatinine, albumin, globulin and bilirubin levels were determined using kits from Randox Laboratories Limited (United Kingdom), which are indicators of the liver and kidney function. Reduced level of total protein, albumin and globulin in diabetic groups compared to non-diabetic groups were reported. Administration of MO reduced creatinine and total bilirubin levels suggestive of ameliorative effects of MO on both the kidney and the liver.

Hepatic enzymes, aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) are markers of damage. Their activities were elevated in hyperglycemic groups indicative of cellular damage but reduced significantly with MO treatment a reflection of nephroprotective and hepatoprotective action of MO.

Inflammatory cytokines such as interleukin-1 α (IL-1 α), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-18 (IL-18), tumour necrotic factor- α (TNF- α) and chemokine monocyte chemotactic protein-1 (MCP-1) concentrations were assessed in liver and kidney

homogenates and serum of rats using MILLIPLEX[®] MAP rat cytokine magnetic bead-based Luminex kit (Merck Millipore, Billerica, MA, USA).

Pro-inflammatory cytokine and chemokine genes play a vital role in the onset and progression of diabetes. Our study provides evidence of an increased concentration of cytokines IL-1 α , IL-6, IL-12, IL-18, TNF- α and chemokine MCP-1 concentrations in the kidney, liver and serum of diabetic rats when compared to the normal control and diabetic control. TNF- α . This is because oxidative stress increases expression of the pro-inflammatory gene by oxidant-mediated activation of transcription factors as well as stress-mediated response. ROS is critical in inflammatory response to oxidants through the upregulation of redox-sensitive transcription factors, alteration of histones acetylation or deacetylation and thus pro-inflammatory gene expression. These parameters were reduced after MO treatment in both non-diabetic and diabetic groups, which may be due to the upregulation of endogenous antioxidants (GSH, SOD, CAT) in response to ROS. The observed decrease in the inflammatory cytokines shows retardation in the onset of diabetic nephropathy and hepatopathy.

Endogenous antioxidant enzymes, SOD, CAT, GPx activities and GSH concentration were evaluated to ascertain the effects of MO on the oxidative status of diabetic and non-diabetic rats. These endogenous antioxidants are biologically synthesised to provide a defence mechanism against ROS-induced cellular damage. The study established that MO increases some enzymatic activities kidney, liver and erythrocytes. SOD catalyses the dismutation of superoxide radical generated from over production of reactive species in a diabetic state to hydrogen peroxide, a more stable product which is degraded to water by other enzymatic reaction (McCune & Johns, 2002). Also, catalase metabolically degrades hydrogen peroxide (H₂O₂) to oxygen and water (Afshari et al., 2007). GSH scavenges hydroxyl radical and singlet oxygen and it is the major antioxidant enzyme in liver cells which detoxifies radicals thereby defending cells against oxidative damage. GPx acts by reducing H₂O₂ and other peroxides using GSH as substrate. This enzyme oxidases GSH to GSSG which can be reduced to GSH by GSH reductase.

In the liver, SOD, CAT GPx activities and GSH decreased significantly in diabetic groups when compared to the normal control. The decreased actions of SOD, CAT, GPx and GSH in diabetic rats may be due to inactivation caused by free radicals. Also, reduced SOD activity in diabetic rats could be due to a rise in H₂O₂ in the liver tissues (Nishikawa & Araki, 2007; Venditti et al., 2015). However, MO increased the activities of these enzymes in the diabetic treated group.

In this study, the activities of CAT, SOD, GPx and GSH concentration in the kidney tissue homogenate of the diabetic groups when compared to normal control. Following treatment with MO, the enzyme activity we elevated in CAT but remained unchanged in SOD, GPx and GSht level (Al-snafi, 2015). Antioxidant enzyme activities (CAT) was high possibly in an attempt to inactivate peroxy radical and superoxide anion converting them to water and oxygen in diabetic rats after treatment.

Similarly, SOD, CAT and GPx activities in erythrocytes were reduced in diabetic rats compared to normal control and increased after MO treatment. GSH concentration increased significantly in diabetic groups when compared to non-diabetic groups and decreased after treatment. Alteration of these antioxidant enzyme activities led to oxidative stress and injury as observed in diabetic rats. MO exerts its antioxidant properties by preventing excessive oxidation of macromolecules such as lipids, proteins, carbohydrates and DNA (Chumark et al., 2008). The antioxidant defence system (SOD, CAT, GPx and GSH) is capable of scavenging and stabilizing free radicals before they attack cells, organs and macromolecules (Chumark et al., 2008).

This study also elucidates MO's ability to reduce, inhibit and alleviate the development of oxidative damage, possibly due to the presence of terpenoids in MO which is involved in the stimulation of beta cells to secrete insulin (Naugler & Karin, 2008). MO exerted its ameliorative effects by reducing oxidative stress, endothelial cell dysfunction, kidney lipid peroxide and increasing antioxidant capability relating to MO's phytochemical composition.

Lipid peroxidation was determined in the kidney homogenate by measuring the conjugated dienes and malondialdehyde. Increased lipid peroxidation was observed in diabetic groups reflecting damage to cell membrane lipids which could lead to altered membrane fluidity, increased permeability of membrane and inactivation of membrane-bound enzyme and receptors (Manohar et al., 2012). A decreased lipid peroxidation was observed after MO treatment. Lipid peroxidation plays a role in enhancing inflammatory mediators through the activation of stress kinases (JNK, MAPK and P38) and redox sensitive transcription factors (NFKB, and AP-1). Evidence also indicates that oxidative stress and pro-inflammatory mediators can alter nuclear histones acetylation allowing access for transcription factor for DNA binding, leading to enhanced pro-inflammatory gene expression. The end product of lipid peroxidation has an adverse effect on vital organs (Huebschmann et al., 2006).

Serum lipid profile; LDL, TC and HDL in diabetic and non-diabetic male rats were assessed. The liver synthesizes LDL by the action of lipolytic enzymes, and increased concentrations lead to cholesterol build-up in the arteries. Excess cholesterol level is harmful to cellular integrity and forms plaque in artery walls. Increased oxidative stress as a result of free radicals is seen in diabetic groups with increased LDL and cholesterol levels culminating in the formation of plaques in the artery walls and reducing the rate of blood flow in diabetic rats. HDL, the good cholesterol converts other forms of cholesterol to bile acid and it is excreted. HDL level was reduced in diabetic groups. The activity of MO leaves extracts was shown in lowered TC and LDL levels, and increased HDL level in our study.

Expression and activity of caspases; apoptotic proteins, in diabetic rats and non-diabetic male Wistar rats were assessed in the kidney and liver. For this study, immunohistochemistry (IHC) was used in the assessment of apoptotic cell death markers in the kidney and liver. The study revealed individual cell's intracellular structure as well as the expression of specific proteins. The expression and repression of caspase 3, caspase 9, BCL-2, NFK β p105 and p53 protein were performed in the kidney and liver of diabetic and non-diabetic rats. The induction of

diabetes with STZ in rats resulted in hyperglycemia which activated the high synthesis of inflammatory proteins, generation of ROS and activation of apoptotic response (Ghosh et al., 2015; Gopalakrishnan et al., 2016).

Increased expression of caspase 3, caspase 9, NFK β , p53 and decreased expression of BCL-2 were observed in the kidney and liver tissues. Diabetic rats exhibited the high intensity where the neural cells were lost with increased excessive cell death, compared to the control and other groups. This may be as a result of cell damage, a major cause of cell death. Similar studies have been reported in animal models (Manna et al., 2010; Peixoto et al., 2015). The improvement observed after treatment with MO may be a resultant effect of the anti-apoptotic and anti-proliferative activities of MO which reduced the expression of these proteins, thus inhibiting the growth of abnormal cells.

Furthermore, histopathology sections of the kidney, liver and pancreas of rats were evaluated. Kidney sections of diabetic rats showed severe renal damage with interstitial nephritis at the cortical area of the kidney and glomeruli haemorrhage which is associated with diabetes. However, administration of MO showed appreciable improvements to these alterations revealing mild vascular congestion of the glomerulus. This result suggests that diabetic nephropathy was significantly reduced after MO treatment. The outcome is consistent with the findings of Kandasamy and Ashokkumar (2013) who reported significant changes in the pathology of the kidney of diabetic rats after treatment.

Liver specimens from diabetic rats revealed severe hepatocyte necrosis at the centrilobular zone when compared to the diabetic treated rats. After treatment of diabetic rats with MO, liver section showed moderate portal congestion and very mild periportal cellular reaction.

Pancreatic sections of diabetic rats showed severe congestion of the major interstitial vessels, thickening of the interstitial vessel and degenerating islets when compared to non-diabetic control group. After treatment of diabetic rats with MO, pancreatic sections of diabetic rats

revealed slightly prominent interstitial connective tissues and rejuvenated islets when compared to diabetic control group. MO is capable of repairing and reversing damage caused by free radicals thereby protecting pancreatic β -cells (Gupta et al., 2005).

MO has shown beneficial effects in pathological conditions in experimental animal models, acting as an antioxidant agent through different mechanisms. This study revealed an insight into the pharmacological effect through its hypoglycaemic, anti-oxidative and dyslipidemic effects in diabetic nephropathy and liver injury resulting from streptozotocin- induced damage in diabetic male Wistar rats. It also demonstrated its potential effects on specific apoptotic proteins and its pathological effects on other selected cells and organs, which could potentially revolutionalise the use of natural products in the treatment and management of diabetes

7.1 Conclusion

The following conclusions can be drawn from this study:

1: Hyperglycemia was successfully induced in the animal model as confirmed in the blood and kidney biomarkers. Oxidative stress, inflammation and nephropathy were observed in diabetic groups while treatment with methanol extract of MO ameliorated the effect. This suggests that MO has hypoglycaemic activity and high antioxidant capacity due to its high polyphenol, flavonoid and flavonol content. MO also enhanced the antioxidant status and reduced lipid peroxidation indicating that it has the potential to be used as an antidiabetic agent in the treatment and management of diabetes.

2: MO methanol leaves extracts showed hepatoprotective, lipid-lowering effect against hyperglycemia and liver damage induced by STZ in rats. MO may be able to lower the risk of cardiovascular diseases with its ability to lower the levels of cholesterol and low-density lipoprotein while increasing high-density lipoproteins.

3: It demonstrated its capability to repair the damaging effects of diabetes in pancreatic β -cells with the potential of proffering beneficial effects to individuals with poor glycemic control. It is our opinion that MO could be taken as a dietary supplement as an anti-hyperglycaemic, antioxidant and anti-inflammatory agent.

4: The study demonstrates the anti-apoptotic properties of MO treatment showing its potency to reduce renal and hepatic damage induced by STZ and to regulate the expression and suppression of specific apoptotic cell death markers.

5: MO leaves have rich antioxidant and phytochemical contents responsible for its medicinal activities that offer beneficial effects as a good nutrient source and to combat numerous diseases.

In summary, the aim of this study was to examine the protective effects of MO leaves extract in streptozotocin (STZ)-induced diabetes in kidney, liver, pancreas and blood of rats. The study further examined specific diabetic, oxidative stress, apoptotic, inflammatory and pathological markers in these tissues. The study revealed that MO leave extract is very effective against streptozotocin-induced diabetes in rats. Treatment with MO almost restored to normal the adverse effects caused by STZ in the blood, kidney, liver and pancreatic markers after a period of six (6) weeks. The results showed that MO extracts have the potency to counteract the formation of free radicals and other reactive species generated by the disease state in the kidney, liver and blood thereby decreasing oxidative stress and preventing cellular damage.

It is evident that MO is able to protect the kidney, liver and pancreas against harmful effects from diabetic complications. This study elucidated the fact that MO can be used as a therapeutic and hypoglycaemic agent in the management of diabetes and the associated complications (nephropathy and chronic liver injury).

7.2 Recommendation

In view of the evidence of the potential effects of MO as revealed in this study, there is still a need for further studies on the standardization of the extracts, isolating polyphenolics compounds in pure form so as to establish the exact mechanism by which MO exerts its antidiabetic activity on the cellular level and the possible pathway involved. Considering the complexity of diabetes, a clinical trial is highly recommended.

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ADDENDUM
RESEARCH OUTPUT

PUBLISHED MANUSCRIPT AND ABSTRACT

- I. Elizabeth I Omodanisi, Yapo G Aboua, Oluwafemi O Oguntibeju. Review Article: Therapeutic potentials and pharmacological properties of *Moringa oleifera* Lam in the treatment of diabetes mellitus and related complications. Tropical Journal of Pharmaceutical Research, 2017; 16 (7)1737-1746.
- II. Elizabeth I Omodanisi, Yapo G Aboua, Novel N Chegou, Oluwafemi O Oguntibeju. Hepatoprotective, anti-hyperlipidemic and anti-inflammatory activities of *Moringa oleifera* in diabetes-induced damage in male Wistar rats. Pharmacognosy Research 2017' 9: 192-187.
- III. Elizabeth I Omodanisi, Yapo G Aboua, Oluwafemi O Oguntibeju. Assessment of the anti-hyperglycaemic, anti-inflammatory and antioxidant activities of the methanol extract of *Moringa oleifera* in diabetes-induced nephrotoxic male Wistar rats. Molecules. Manuscript number: Molecules, 2017, 22(4) 439-455.
- IV. Elizabeth I Omodanisi, Yapo G Aboua, Oluwafemi O Oguntibeju. *Moringa oleifera* leaves modulate inflammation and oxidative stress in the liver, plasma and erythrocytes of streptozotocin-induced diabetes in rats. Published in the book of abstract at the 1st SOMPED conference on Medicinal Plants and Economic Development. August 14th - 17th, 2016, Johannesburg, South Africa.
- V. Oluwafemi O Oguntibeju, Elizabeth Ife Omodanisi, and Guillaume Aboua, *Moringa oleifera* significantly reduced hyperglycaemic effects in STZ-induced diabetic rats. Presented at the 14th International conference and exhibition on Targeting Diabetes and Novel Therapeutics, October 17th-18th, 2016, Kuala Lumpur, Malaysia. Abstract published in Journal of Diabetes & Metabolism, 7(9): 1-2.

MANUSCRIPT IN PRESS

- I. Elizabeth I Omodanisi, Yapo G Aboua, Oluwafemi O Oguntibeju. Review Article: Therapeutic potentials and pharmacological properties of *Moringa oleifera* in the treatment and management of Diabetes Mellitus and other related complications. Tropical Journal of Pharmaceutical Research. ISSN: 1596-5996.

CONFERENCE ATTENDANCE

- I. 2nd International Congress of the World Public Health Nutrition Association at the University of the Western Cape (UWC), Cape Town South Africa. August 2016.

- II. Cape Peninsula University of Technology Post Graduate Research Conference, Bellville, South Africa. To enhance postgraduate students' competency, skill and knowledge in presentation and publication. November 2014.