



**ANTIDIABETIC AND HEPATOPROTECTIVE EFFECTS OF *Moringa oleifera* LEAF  
EXTRACTS IN STREPTOZOTOCIN-INDUCED DIABETES IN RATS.**

BY

TAMBWE WILLY MUZUMBUKILWA

217040227

**2018**

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BY

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A Dissertation submitted in fulfillment of the requirements of the degree of Master of Medical Science (Pharmacology), in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000. South Africa.

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University of KwaZulu-Natal.

As the candidate's supervisors, we have approved this dissertation for submission.

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## PREFACE

The experimental work described in this dissertation was carried out in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Science, University of KwaZulu-Natal, Westville, from January 2017 to October 2018, under the supervision of Dr. Owira P.M.O and Dr. Manimbulu Nlooto.

This dissertation is presented in a manuscript format and consists of three chapters.

**Chapter one:** This chapter provides a general introduction, comprehensive literature review, stating the epidemiology and prevalence of diabetes mellitus. This chapter also provides the pathophysiological mechanisms of diabetes-induced liver damage. The chapter also includes the medicinal use of *Moringa oleifera* in managing diabetes. General objectives, materials, and methods used in the study are also stated in this chapter.

**Chapter two:** This chapter provides a manuscript for publication presented in the required format of the journal.

Manuscript title: Hepatoprotective effects of *Moringa oleifera* Lam (Moringaceae) leaf extracts in streptozotocin-induced diabetes. Journal of Functional Foods.

Authors: Muzumbukilwa W.T., Nlooto M., Owira P.M.O.

**Chapter three:** This is a chapter which describes the general discussion, conclusion, and recommendations.

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## DECLARATIONS

### DECLARATION 1 – PLAGIARISM

I, **Tambwe Muzumbukilwa Willy** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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## **DECLARATION 2 – MANUSCRIPT PUBLICATION**

**Article title 1: Hepatoprotective effects of *Moringa oleifera* Lam (Moringaceae) leaf extracts in streptozotocin-induced diabetes.**

Authors: Muzumbukilwa W.T., Nlooto M., Owira P.M.O.

Submitted to the Journal of Functional Foods.

**Supplementary Paper: Mapping the evidence of hepatoprotective properties of *Moringa oleifera* from sub-Saharan African countries: a systematic review protocol.**

Authors: Muzumbukilwa W.T., Kadima M.G., Nlooto M., and Owira P.M.O.

Submitted to BMC Systematic reviews.

These articles are presented in the required format of the journal.

Article 1 describes the general findings and discussion of the results of the study.

### **CONFERENCES:**

1. Oral presentation "Hepatoprotective effects of *Moringa oleifera* Lam (Moringaceae) leaf extracts in streptozotocin-induced diabetes " Echohealth international-African chapter conference, Durban, South Africa, 13-14 November 2018.

**Signed:**

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## LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AGEs	Advance Glycation End products
ALAT	Alanine Aminotransaminase
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ANG II	Angiotensin II
ANOVA	Analysis of Variance
ARI	Aldose reductase inhibitors
ASAT	Aspartate Aminotransaminase
AUC	Area Under the Curve
BMI	Body Mass Index
BRU	Biomedical Resource Unit
BW	Body Weight
CCL4	Carbon Tetrachloride
CD4	Cluster of differentiation4
CD8	Cluster of differentiation8
DCM	Diabetic Cardio Myopathy
DKA	Diabetic ketoacidosis
DM	Diabetes Mellitus
DPP-4	Dipeptidyl peptidase
FBG	Fasting Blood Glucose

FFA	Free fatty acids
GAD	Glutamic acid decarboxylase
GC-MS	Gas Chromatography-mass spectrometry
GGT	Gamma-Glutamyltransferase
GLP1	Glucagon-Like Peptide-1
GLUT2	Glucose transporter-2
GLUT4	Glucose transporter-4
GTT	Glucose Tolerance Tests
HbA1C	Hemoglobin A1C test
HDL	High-density lipoprotein
IDF	International Diabetes Federation
LDH	Low-density lipoprotein
LPL	Lipoprotein lipase
LPO	Lipid Peroxidation
MO	<i>Moringa oleifera</i>
NADH	Nicotinamide Adenine Dinucleotide
NAFLD	Nonalcoholic fatty liver disease
NSAID	Nonsteroidal anti-inflammatory Drugs
OGTT	Oral Glucose Tolerance Test
PKC	Protein kinase c
PTB	Phenacyl Thiazolium Bromide
RAAS	Renin-Angiotensin-Aldosterone System

RBX	Ruboxistaurin
RNS	Reactive Nitrogen Species
RPM	Revolutions per minute
SGOT	Serum Glutamate-oxaloacetate
SGPT	serum glutamic-pyruvic transaminase
SOD	Superoxide Dismutase
STZ	Streptozotocin
T1DM	Type1 Diabetes Mellitus
T2DM	Type2 Diabetes Mellitus
TBARS	Thiobarbituric Acid Reactive Substance
TCF7L2	Transcription factor 7-like 2
UKZN	University of KwaZulu-Natal
UNE	Ulnar Neuropathy at the Elbow
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoproteins
WHO	World Health Organization
$\beta$	Beta

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## ABSTRACT

### Introduction

Diabetes Mellitus is one of the major causes of degenerative diseases worldwide. Long term complications of diabetes include hepatic injury characterized by cirrhosis, inflammation, apoptosis, and microvascular and macrovascular aberrations. Mechanisms by which diabetes induces liver damage include the development of lipotoxicity-induced mitochondrial dysfunction and activation of inflammatory pathways that lead to progressive liver damage. Previous studies have reported that the leading cause of death in patients with diabetes mellitus is chronic liver disease. The liver is a metabolically active organ involved in many vital life functions. It performs many activities that are critical for survival. Due to its important activities, the liver is exposed to a number of insults and is one of the body's organs most subject to injury.

Despite considerable progress in modern medicine, there are very few therapeutic agents that can protect the liver from hyperglycemia-induced oxidative damage and restore normal liver functions. As a result, the search for novel therapies that would be cheaper and effective in the management of liver diseases is paramount. *Moringa oleifera* (MO) is a multipurpose plant traditionally used for its medicinal and nutritional properties in many countries, especially in Durban, KwaZulu-Natal/ South Africa where the material for this study has been harvested. It has been shown to possess antihyperglycemic, antioxidant and anti-inflammatory properties and could possibly prevent liver injury. This study, therefore, investigated whether MO leaf extracts could mitigate hepatotoxicity associated with diabetes mellitus.

### Methods

Male Wistar rats (250-300 g) were divided into six groups (n=7). Group A was orally treated daily with 3.0 ml/kg body weight (BW) of distilled water; group C was similarly treated with MO (500 mg/kg/BW) daily. Groups B, D, E, and F were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ) (45 mg/kg/BW in 0.1M citrate buffer, pH4.5). Diabetes was confirmed 3 days later. Additionally, group D was treated with subcutaneous insulin (2 U/kg/BW, bid) while groups E and F were orally treated daily with MO 250 mg/kg/BW and 500 mg/kg/BW, respectively. Glucose tolerance tests (GTT) were done on day 47 of the animal treatment. After an

overnight fast for 8 hours, rats in all groups were intraperitoneally dosed with a D-glucose solution (3.0 g/kg BW) in 0.9% normal saline. This solution was prepared by dissolving 45 g of D-glucose anhydrous in 60 ml distilled water (0.75 g/ml). Blood glucose concentrations were measured by tail pricking at 0, 30, 60, 90, and 120 minutes, using glucometer (OneTouch select®; Lifescan Inc., Milpitas, California, USA). On day 54 of treatment, animals were sacrificed by halothane overdose. Blood was collected by cardiac puncture in heparinized tubes then separated into plasma and stored at -80°C for further biochemical analysis. Livers were excised, snap-frozen in liquid nitrogen and similarly stored for histological analysis.

**Results:** Diabetic animals had significantly ( $p < 0.05$ ) elevated Fasting Blood Glucose (FBG) and reduced insulin levels compared to controls. Treatment with either insulin or MO significantly ( $p < 0.05$ ) reduced FBG compared to non-treated diabetic rats. Treatment with 500 mg/kgBW significantly reduced FBG compared to treatment with 250 mg/kgBW. Calculated Areas-Under-the Curve (AUCs) from OGTT suggested that untreated diabetic rats exhibited glucose intolerance but treatment with either insulin or MO extracts significantly ( $p < 0.05$ ) reversed this. Liver function tests defined by Aspartate Aminotransaminase (ASAT), Alanine Aminotransaminase (ALAT), gamma-glutamyl aminotransaminase (GGT) and albumin were significantly ( $p < 0.05$ ) elevated in untreated diabetic group but treatment with either insulin or MO extracts significantly ( $p < 0.05$ ) reversed this. Treatment with 500 mg/kgBW of MO significantly ( $p < 0.05$ ) reduced GGT levels compared to treatment with 250 mg/kgBW. Untreated diabetic, unlike MO-treated rats, exhibited degeneration of hepatocytes and inflammatory cells infiltration with the fragmentation of the nucleus and cell lysis, necrotic hepatocytes, hepatic vein congestion, and vesicular cytoplasm compared to normal controls.

**Conclusion:** This study has shown that methanolic leaf extracts of MO have dose-dependent antidiabetic effects. Liver function tests (ASAT, ALAT, GGT) and albumin were significantly elevated in untreated diabetic rats than those treated with MO extracts. This may justify the hepatoprotective effects of MO extracts in streptozotocin-induced diabetic rats.

**Keywords:** *Moringa oleifera*, hepatocytes, streptozotocin, diabetes.

## CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

### 1.1 DIABETES MELLITUS

#### 1.1.1 Epidemiology of Diabetes Mellitus.

In 2017, the International Diabetes Federation (IDF) estimated that 425 million adults aged between 20 and 79 years were living with diabetes worldwide (Ogurtsova *et al.*, 2017). Globally, 422 million people were living with diabetes in 2014 and 1.6 million deaths were attributed to diabetes (World Health Organization, 2016). IDF estimates that 75% of people with diabetes live in low-and-middle-income countries (Whiting *et al.*, 2011). Total global health expenditure due to diabetes in 2017 was estimated at 727 billion US dollars (Ogurtsova *et al.*, 2017).

The National Diabetes Statistics Report estimates that 30.3 million people with diabetes (9.4% of the USA population) including 23.1 million people diagnosed and 7.2 million people undiagnosed were living with diabetes in the United States in 2017 (Centers for Disease Control and Prevention, 2017).

Public health burden of diabetes in Africa has been recognized (Motala *et al.*, 2003). The prevalence of diabetes increased from 0-1% in the 1980s to 20% in the 2000s and is predicted to be affecting between 18.6 to 23.9 million Africans by the year 2030 (Mbanya *et al.*, 2010). More so, 14.2 million Africans were estimated to be living with diabetes in 2015, and this was projected to increase to 34.2 million by 2040 (Mutiyambizi *et al.*, 2018). It has further been estimated that 15.5 million adults aged 20-79 years were living with diabetes in Africa in 2017, representing a regional prevalence of 3.3% (Ogurtsova *et al.*, 2017).

In South Africa, 3.5 million people (approximately 6% of the total population) are living with diabetes and many others remain undiagnosed (World Health Organization, 2016). The high incidence of T2DM in South Africa, like in the rest of the world has been attributed to sedentary lifestyle, lack of physical activities and increased urbanization (Menghani *et al.*, 2010). In the year 2000, the South African National Burden of Disease Study reported that diabetes was the tenth leading cause of morbidity and mortality, accounting for about 2.6% of all deaths, which represents 20,000 deaths or 4.3% of the total population (Bradshaw *et al.*, 2007).

### 1.1.2 Definition and classification of diabetes mellitus

Diabetes mellitus is a disorder of carbohydrate, lipid and protein metabolism, characterized by high blood glucose levels resulting from defects in insulin secretion, insulin action, or both. Long-term complications of untreated DM include cardiomyopathy, nephropathy, neuropathy and retinopathy (Alberti and Zimmet, 1998). Classical symptoms of DM include polydipsia, polyuria, blurring of vision and weight loss. As reported by the American Diabetes Association, 4 categories of DM are recognized: Type 1 Diabetes mellitus, Type 2 Diabetes mellitus, gestational diabetes and diabetes due to secondary disease processes or drugs. Maturity-onset diabetes of the young (MODY) is a rare form of diabetes caused by mutations in nuclear transcription factors and glucokinase genes which result in pancreatic  $\beta$ -cell dysfunction in the production of insulin hormone (Cydulka and Maloney., 2002; Navale, 2017).

#### 1.1.2.1 Type 1 Diabetes

Type 1 diabetes mellitus (T1DM) is autoimmune-mediated destruction of pancreatic  $\beta$ -cells. The dendritic cells and macrophages in the pancreatic islets possess autoantigens and present them to autoreactive CD4+ T cells. CD4+ T cells secrete cytokines which can activate cytotoxic T cells (CD8+). These cells activate macrophages and other T cells leading to beta cells destruction ultimately causing absolute insulin deficiency which predisposes individuals to ketoacidosis (Lambert *et al.*, 2004). Genetic and environmental factors have been identified as leading for the autoimmune destruction of the  $\beta$ -cells. Human leukocyte antigen (HLA) is a common genetic risk factor associated with the development of diabetes (Nokoff and Rewers, 2013). Environmental factors involved in the pathogenesis of T1DM include parasites, viruses, and bacteria which mediate the direct infection of the pancreatic  $\beta$ -cells or shape the immune system to mutually benefit the parasite and the host (David *et al.*, 2004).

T1DM leads to complete pancreatic  $\beta$  cell destruction and eventually all patients with T1DM will require insulin therapy to maintain glycemic control. Patients with T1DM are rarely obese and are prone to other autoimmune conditions such as Grave's or Addison's disease (Krzewska and Ben-skowronek, 2016).

All patients with T1DM present with classical symptoms of hyperglycemia and signs of ketoacidosis (David, M. and Nathan., 2009). When the diagnosis is uncertain, other tests can be used including the measurement of plasma C-peptide levels or markers of immune destruction such as islet cell antibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD) and autoantibodies to tyrosine phosphatases IA-1 and IA-2 (Casqueiro et al., 2012). T1DM usually develops in younger and teenage population (adolescent) although it can also occur in adults (Devendra *et al.*, 2004). It has been shown that human leukocyte antigen chromosome 6 is a genetic predisposition that is linked to T1DM (Lie *et al.*, 1999). Type 1 diabetes accounts for 10% of all diagnosed cases of diabetes in adults worldwide (Adeeyo *et al.*, 2013).

### 1.1.2.2 Type 2 Diabetes

T2DM is insulin independent and develops when there is impaired glucose tolerance, obesity, dyslipidemia, insulin resistance (IR), increased hepatic glucose production, pancreatic  $\beta$ -cell dysfunction and hyperglycemia (Wu *et al.*, 2014). Insulin usually controls glucose homeostasis by stimulation of glucose uptake into peripheral tissues, and by signaling the liver to take up glucose and store it as glycogen. Production of advanced glycation end products (AGE), increased stimulation of hemodynamic regulation systems such as the renin-angiotensin system (RAS) and elevated production of reactive oxygen species (ROS) which are associated with chronic hyperglycemia plays a pivotal role in the initiation of diabetic vascular complications (Jakus, 2004). However, the pancreas lacks antioxidant defense systems antioxidant (due to the absence of glutathione peroxidase-1 gene) against ROS (Rahal *et al.*, 2014). In this regard, dyslipidemia is initiated by insulin resistance which activates lipoprotein lipase (LPL) in adipocytes. The lipoprotein lipases catalyze the release of free fatty acids (FFA) which are taken up by hepatocytes leading to the production of triglycerides. Triglycerides then stimulate the production of very low-Density Lipoproteins (VLDL) which contribute to the increased risk of cardiovascular diseases (Ginsberg, H. and Sung, H, 2012). The specific causes of Type 2 diabetes are not well defined, but some factors are incriminated such as environmental, genetic as well as lifestyle changes (Zheng *et al.*, 2017). In "prediabetes", blood glucose levels are higher than normal but not yet high enough to be diagnosed as diabetes. Research has shown that some long-term damage to the body organs especially the heart and circulatory system, may already be occurring during prediabetes (Silva *et al.*, 2008). Cardiovascular disease is the foremost killer of patients with diabetes.

The metabolic syndrome is a clustering of risk factors that often accompany obesity and associated with increased risk for both cardiovascular diseases and type 2 diabetes (Ford *et al.*, 2008). This cluster of risk factors led to diabetes and cardiovascular diseases. Such factors include hypertension, insulin resistance, defective glucose metabolism (impaired fasting glycemia or glucose intolerance), increased LDL-c, triglycerides, obesity and low HDL-c concentrations (Paula *et al.*, 2013a). European Group for Study of Insulin Resistance (EGIR), International Diabetes Federation (IDF), American Association of Clinical Endocrinologists (AACE), WHO clinical criteria (WHO 1999) and the National Cholesterol Education Program Adult Treatment Panel III (NCEP/ATP III) have defined the criteria classification of metabolic syndrome (Table 1) (Paula *et al.*, 2013b). These groups have a harmonized definition of the metabolic syndrome with criteria defined in Table 1 below.

**Table 1:** The definition of metabolic syndrome by different criteria (Alberti and Zimmet., 1998; Einhorn *et al.*, 2003). Legend: BMI: body mass index; DM: diabetes mellitus; FBG: fasting blood glucose; IFG; impaired fasting glucose; IGT: impaired glucose tolerance; IR: insulin resistance; HDL: high-density lipoprotein; TG; triglyceride; WC: Waist circumference.

Clinical measures	World of Health Organization (1998)	European Group for Study of Insulin Resistance (1999)	National Cholesterol Education Program Adult Treatment Panel (2001)	American Association of Clinical Endocrinologists (2003)	International Diabetes Federation (2005)
Insulin resistance	IGT, IFG, T2DM, or Reduced insulin Sensitivity	Plasma insulin >75th percentile	None	IGT or IFG	None
Body weight	Male: waist-tohip > 0.90 Female: Waistto-hip > 0.85 and/or BMI > 30 kg/m <sup>2</sup>	Male: WC ≥ 94 cm Female: WC ≥ 80 cm	Male: WC ≥102 cm Female: WC ≥ 88 cm	BMI ≥ 25 kg/m <sup>2</sup>	Increased WC (populace specific)

Dyslipidemia	TGs $\geq$ 150 mg/dl and/or HDL-c $<$ 35 mg/dl in males or $<$ 39 mg/dl in females	TGs $\geq$ 150 mg/dl and/or HDL-c $<$ 39 mg/dl in males or females	TGs $\geq$ 150 mg/dl HDL-c $<$ 40 mg/dl in males or $<$ 50 mg/dl in females	TGs $\geq$ 150 mg/dl and HDL-c $<$ 40 mg/dl in males or $<$ 50 mg/dl in females	TGs $\geq$ 150 mg/dl HDL-c $<$ 40 mg/dl in males or $<$ 50 mg/dl in females or on HDL-c treatment
Blood pressure	$\geq$ 140/90 mm Hg	$\geq$ 140/90 mm Hg or on hypertension treatment	$\geq$ 130/85 mm Hg	$\geq$ 130/85 mm Hg	$\geq$ 130 mm Hg systolic or $\geq$ 85 mm Hg diastolic or on hypertension treatment
Glucose Metabolism	IGT, IFG, or T2DM	IGT or IFG (None diabetes)	$>$ 110 mg/dL (comprises diabetes)	IGT or IFG (None diabetes)	$\geq$ 100 mg/dL (comprises diabetes)

The major causes of metabolic syndrome include genetic and environmental factors which influence both lipid and glucose metabolism. An estimated 20-25% of the adult individuals have been clinically diagnosed with metabolic syndrome globally (Moreira et al., 2014).

Genes that control the expression of lipases, adiponectin, uncoupling proteins,  $\beta$ -Adrenergic receptors, PPAR- $\gamma$ , glycoprotein PC-1, skeletal muscle glycogen synthase 1, IRS-1, Calpain-10, CD36, fatty acid binding protein-2, apolipoprotein E, upstream transcription factor 1 and 11  $\beta$ -hydroxysteroid dehydrogenase type 1 have associated with increased incidence of metabolic syndrome (Stancakova and Laakso., 2014). Pro-inflammatory cytokines like tumor necrosis factor (TNF) and Interleukin 1- $\beta$  (IL1- $\beta$ ), C-reactive protein and neuropeptide Y(NPY), leptin receptor, pro-opiomelanocortin (POMC) and melanocortin receptors are also involved in metabolic syndrome (Groop, 2007).

Environmental factors that lead to metabolic syndrome include physical inactivity, obesity, smoking and aging, which have been suggested to induce insulin resistance, cardiovascular disorders, inflammation, hyperinsulinemia and endothelial dysfunction (Takashi *et al.*, 2006). These factors are associated with dysfunctions of adipocytes leading to altered production of

hormones and cytokines such as interleukin-6 (IL-6), TNF- $\alpha$ , resistin, adiponectin, and leptin (Takashi *et al.*, 2006).

### 1.1.2.3 Gestational Diabetes

Gestational diabetes is defined as glucose intolerance with first recognition pregnancy. Women who are overweight, had gestational diabetes before or have a family history of diabetes are at a higher risk of developing gestational diabetes. Untreated gestational diabetes may cause problems to the fetus such as breathing difficulties and low blood sugar levels. Gestational diabetes is associated with increased risk of maternal and fetal complications. Fetal macrosomia is a common complication of diabetes in pregnancy. Pregnant woman of a macrosomic fetus is at increased risk for preeclampsia, labor abnormalities, severe perineal lacerations, risks of preterm birth and cesarean section. The fetus is at risk for stillbirth, intracranial hemorrhage, shoulder dystocia and malformations (Susa JB, 1988). The neonate is at risk for hyperbilirubinemia, hypocalcemia, hypoglycemia, hypomagnesemia, polycythemia and neonatal cardiomyopathy (Macintosh MC *et al.*, 2006). Fetal macrosomia is caused by fetal hyperinsulinemia that occurs as a physiological response to maternal hyperglycemia (Moreli *et al.*, 2016).

Later in life, the baby may be at an increased risk for obesity and T2DM for the rest of their lives (Reece, 2010).

### 1.1.3 Pathophysiology and major risk factors for T2DM

Type 2 diabetes is caused by a combination of genetic factors related to impaired insulin secretion and insulin resistance and environmental factors such as lack of exercise, aging, stress as well as obesity. Impaired insulin secretion and insulin resistance contribute jointly to the development of pathophysiological conditions. Although the pathogenesis of T2DM has been attributed mainly on insulin and beta-cell dysfunction, an abnormal increase in alpha-cell function which results in hyperglucagonemia and consequent hepatic glucose production has long been recognized as a contributory factor to hyperglycemia in diabetic patients. Postprandial state in T2DM patients is characterized by elevated fasting concentrations of glucagon, impaired glucose-induced glucagon

suppression, and disrupted insulin–glucagon interaction against those of healthy subjects. Alterations in the cross-talk between beta- and alpha-cells may underlie diminished mass of insulin pulses and hyperglucagonemia in T2DM patients (Menge *et al.*, 2011). Because of resistance to the actions of insulin and reduced insulin secretion, tissues such as skeletal muscles, liver, and adipose tissues are affected, leading to elevated blood glucose levels (Stumvoll *et al.*, 2005). In T2DM, insulin resistance plays an important role due to increased gluconeogenesis and decreased glucose uptake in the skeletal muscles and adipose tissues at a regular plasma insulin level. In addition,  $\beta$ -cell dysfunction results in reduced insulin release, which is not enough for maintaining normal glucose levels (Weyer *et al.*, 1999).

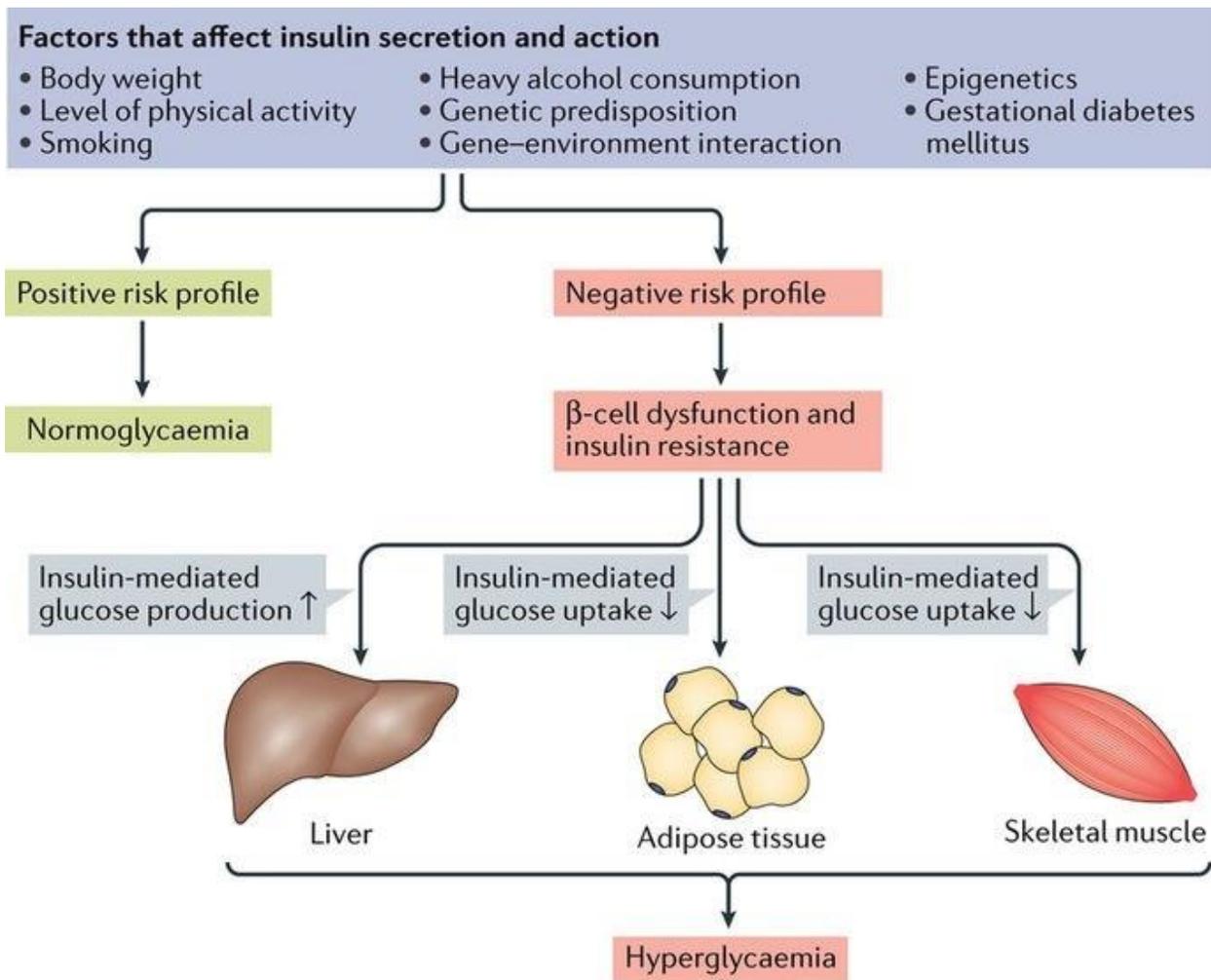


Figure 1: Consequences of insulin action/inaction in Type 2 diabetes mellitus.

Insulin secretion from the  $\beta$ -cells in the pancreas normally suppresses gluconeogenesis and increases glucose uptake by skeletal muscles and adipose tissues. Diminished pancreatic  $\beta$ -cells function leads to hyperglycemia (Zheng *et al.*, 2017).

### **Major risk factors for T2DM**

There are many factors that contribute to T2DM such as:

- Older age
- The family history of type 2 diabetes mellitus (T2DM)
- Low socioeconomic status
- Genetic factors (for example, carrying risk alleles in the *TCF7L2* gene)
- Components of the metabolic syndrome (increased waist circumference, increased blood pressure, increased plasma levels of triglycerides, low plasma levels of HDL cholesterol and small, dense LDL cholesterol particles)
- Overweight or obese ( $\text{BMI} \geq 25 \text{ kg/m}^2$ )
- Abdominal or central obesity (independent of BMI)
- Cigarette smoking
- Sedentary lifestyle
- History of gestational diabetes mellitus or delivery of neonates  $>4$  kg in weight.
- Some medications, such as statins, thiazides, and beta-blockers
- Psychosocial stress and depression (Spiegel *et al.*, 2005).

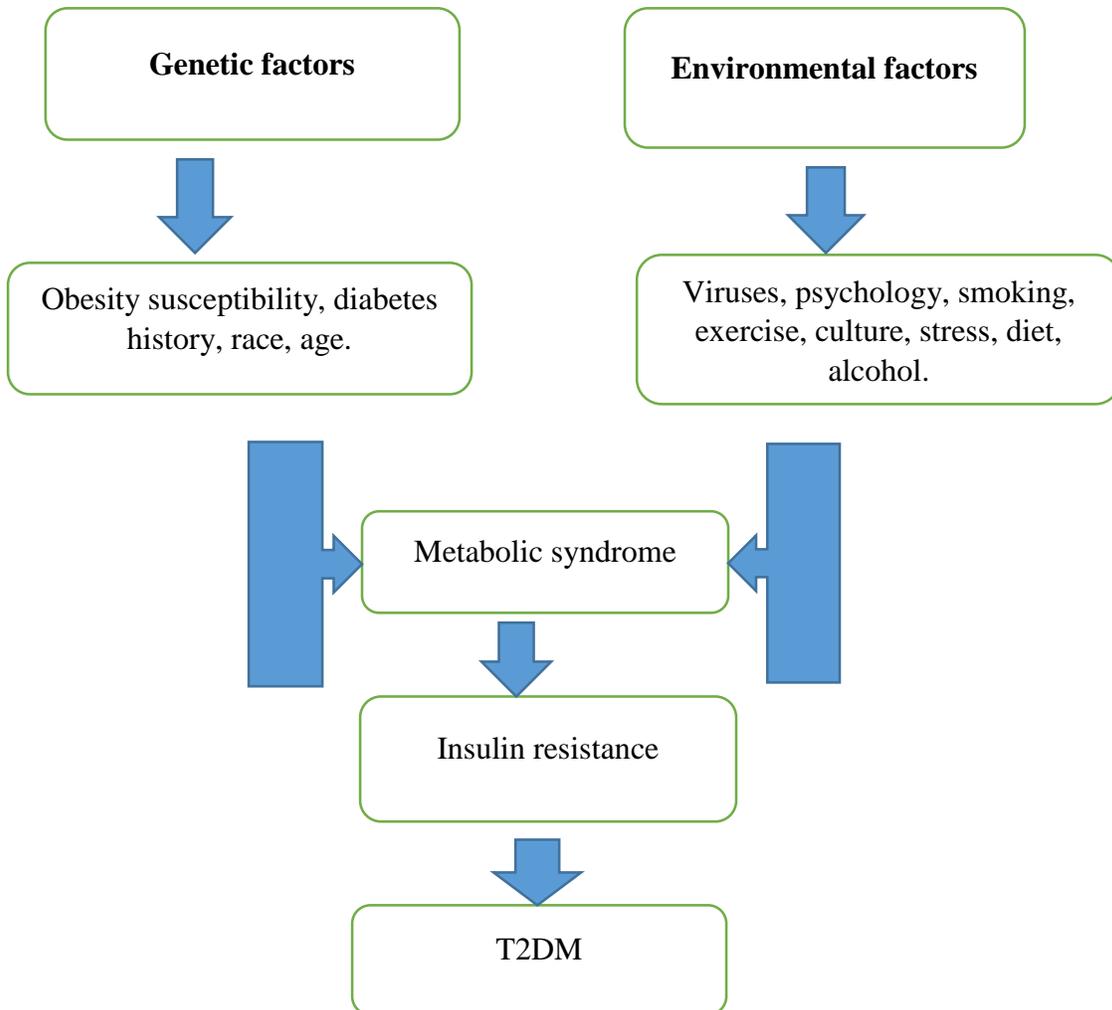


Figure 2: **Genetic and environmental factors of T2DM.**

#### 1.1.4 Complications of diabetes.

Persistent hyperglycemia leads to both acute and chronic complications. Major tissues affected by hyperglycemia are nervous system, the retina of eye, kidney, adipose tissue, liver and pancreatic  $\beta$  cells. T1DM and T2DM both result in long term macro and microvascular complications. Microvascular complications include diabetic retinopathy, neuropathy, and nephropathy, while macrovascular complications include coronary artery diseases, peripheral vascular disease, and stroke (Casqueiro, et al., 2012). Diabetic Ketoacidosis (DKA) is one of the major acute complications of T1DM characterized by ketoacidosis and electrolyte imbalances, caused by absolute lack of insulin; characterized by dehydration and lipolysis (Cydulka *et al.*,2002). In DKA, there is a deficiency of insulin, which results in increased plasma catabolic hormones, that cause overproduction of glucose (Aronson and Ferner, 2003). In renal tubes, excess glucose draws water and electrolytes such as calcium, magnesium, potassium, and sodium into the urine, from the circulation. This osmotic diuresis causes dehydration and other electrolyte imbalances associated with diabetic ketoacidosis (Cydulka *et al.*,2002).

##### 1.1.4.1 Diabetic Retinopathy.

Diabetic retinopathy is one of the most common complications of untreated DM and leads to visual impairment and blindness in adults aged between 20-74 years ( Lilly *et al.*, 2006; Forbes and Cooper, 2013). Lesions in the retina cause alterations of retinal structures such as changes in vascular permeability, capillary degeneration, capillary microaneurysms, and neovascularization by increased formation of new blood vessels (Lilly *et al.*, 2006). Functional impairment of the neural retina alters retinal electrophysiology which results in an inability to differentiate between colors (Forbes & Cooper, 2013). The development and progression of DM are dependent on the severity and duration of hyperglycemia.

Clinically, diabetic retinopathy is divided into non- proliferative and proliferative disease phases. In the initial phase, hyperglycemia can lead to intramural pericyte (cells that wrap around the endothelial cells of capillaries and venules throughout the body) death and thickening of the basement membrane, which contribute to changes in the integrity of blood vessels within the

retina, altering the blood-retinal barrier and vascular permeability (Watkins, 2003). During this primary phase of nonproliferative diabetic retinopathy (NPDR), no visual impairment is noticed. Degeneration or occlusion of retinal capillaries is associated with failing prognosis, which is most likely the result of ischemia followed by subsequent release of angiogenic factors including those related to hypoxia (Osborne N.et al., 2004).

In the proliferative phase neovascularization and accumulation of fluid within the retina, called macular edema, contribute to visual damage. In more severe cases, there can be bleeding with associated distortion of the retinal architecture including the development of a fibrovascular membrane which can subsequently lead to retinal detachment (Sikorski *et al.*, 2013).

Diabetic retinopathy can be prevented with early diagnosis and treatment.

In order to maintain glycemic control and blood pressure, treatments with an injection of the steroid triamcinolone, laser photocoagulation, and vascular endothelial growth factor (VEGF) antagonists into the eye, and vitrectomy, to remove the vitreous (Forbes & Cooper, 2013). However, newer agents have been developed (Table 2).

**Table 2: Potential therapies in the treatment of diabetic retinopathy** (Nawaz *et al.*, 2013).

<b>Targets</b>	<b>Drug</b>
Inhibitor of AGE formation	Pyridoxamine, Aminoguanidine, OPB-9195, ALT-946, ALT-711, LR-90, N-PheracylThiazolium Bromide (PTB), Alagebrium.
Protein kinase C (PKCs) inhibitors.	Ruboxistaurin (RBX), PKC412.
Aldose reductase Inhibitors (ARIs).	Sorbinil, Tolrestat, Epalrestat, Lidorestat, Zenarestat, Ranirestat, Ponalrestat, Zopolrestat, ARI-809, Fidarestat.
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Aspirin, Nepafenac, Sodium salicylate, Sulfasalazine, Baicalein, Genistein, Nepafenac, Celecoxib

#### 1.1.4.2 Diabetic Neuropathy

Diabetic neuropathy is characterized by a progressive loss of nerve fibers affecting both the autonomic and somatic divisions. It is defined by impaired damage healing due to a decrease in oxygen in tissues because of glycated hemoglobin and altered immune system (Schalkwijk, 2008). As with other microvascular complications, the risk of developing diabetic neuropathy is relative to chronic glucose intolerance even though some individuals may have a genetic predisposition (Dobretsov *et al.*, 2007).

There are two classes of diabetic neuropathies: diabetic polyneuropathy in which hyperglycemia causes diffuse damage to the peripheral nervous system; and mononeuropathy, where nerve damage is focal (Boulton, A.J., 2005). Polyneuropathy is the most common form of diabetic neuropathy and may present as cranial, autonomic neuropathy and peripheral motor neuropathy. While mononeuropathies include Carpal Tunnel Syndrome (CTS) and Ulnar Neuropathy at the Elbow (UNE). Both polyneuropathy and mononeuropathy play significant roles in sensory and motor deficits and are both associated with debility in patients (Zochodne, 2007).

The precise nature of the injury to the peripheral nerves from hyperglycemia is not known but it may be related to mechanisms such as polyol accumulation, injury from AGEs, and oxidative stress. AGEs play significant roles in sensory and motor deficits; they induce expression of inflammatory genes resulting in neurologic dysfunction and altered pain sensation (Herold *et al.*, 2007).

Up to 80% of amputations occur after foot ulceration or injury, which can result from diabetic neuropathy (Doupis, J. and Alexiadou, K., 2012).

Neurological dysfunction can affect gastrointestinal system, cardiovascular system or reproductive system; and can be manifested by neuropathy signs such as bladder dysfunction, constipation, diarrhea, erectile dysfunction, exercise intolerance, resting tachycardia, silent ischemia, and sudden cardiac arrest (Kempler *et al.*, 2011).

Current management of diabetic neuropathy is mainly based on symptoms. Analgesics are used to tranquilize pain. However, in severe conditions, opiates such as amitriptyline, gabapentin, duloxetine, and pregabalin may be required (Boulton *et al.*, 2005). Management of peripheral neuropathy requires holistic approach and patients should be informed about the importance of foot care and regular checkups (Navale, 2017). Autonomic neuropathy manifests as gastroparesis

and erectile dysfunction and has devastating effects on lifestyle. Cases of gastroparesis are usually treated with domperidone, erythromycin, and metoclopramide, as well as dietary changes. However, severe cases may require implantation of electrodes to stimulate gastric contractions (Boulton, A.J. et al., 2005).

#### 1.1.4.3 Diabetic Nephropathy (DN).

Diabetic nephropathy is defined as “microalbuminuria or macroalbuminuria and impaired renal function characterized by elevated serum creatinine, reduced creatinine clearance, or increased glomerular filtration rate”(Navale, 2017).

Predisposing factors to DN include hemodynamic changes, metabolic changes, as well as environmental and genetic factors. Elevated serum AGEs levels that occur as a result of hyperglycemia have been found in nephropathic kidneys (Lilly *et al.*, 2006). Hemodynamic changes occur early in diabetic nephropathy and are often characterized by glomerular hyperfiltration (Forbes *et al.*, 2013). DN is the leading cause of chronic kidney disease and serves as a risk factor for diabetic cardiomyopathy (Ronco et al., 2010). DN is defined by the presence of proteinuria  $>0.5$  g/24 h and is categorized into stages based on urinary albumin excretion (ADA, 2005). The cutoff values (timed, 24-h, and spot urine collection) for the diagnosis of microalbuminuria and macroalbuminuria is 30-299 mg/24h and  $\geq 300$  mg/24h respectively (ADA, 2004). DN is clinically characterized by the thickening of the glomerular basement membrane, and accumulation of matrix material in the mesangium. This is followed by nodular deposits, and glomerulosclerosis. At the terminal stage, the glomeruli are lost, hence the function of the kidney declines (Marshall, 2016).

Treatment is focused on control in blood glucose, dyslipidemia, hypertension, microalbuminuria, smoking cessation, and weight management. However, anemia, acidosis and bone disease, as well as malnutrition are common conditions secondary to renal disease, which also need to be treated (Levey and Coresh, 2012). Reduction in blood pressure is vital in the prevention and treatment of DN. The target blood pressure of 120-130 mm Hg is recommended for patients of T2DM to prevent loss of renal function and prevention of diabetic nephropathy ( Grassi, G. et al., 2016). In DN initiation of treatment is recommended for patients with urinary albumin excretion of  $>30$  mg/day.

Treatment of DN includes blockade of Renin-Angiotensin-Aldosterone System (RAAS) with ACE inhibitors or angiotensin receptor blocker drugs with maximum tolerable doses. Treatment with ACE inhibitors in clinical studies has demonstrated a reduction in the progression of nephropathy in diabetic patients (Lewis et al., 1993).

#### 1.1.4.4 Diabetic cardiomyopathy (DCM).

Diabetic cardiomyopathy is defined as functional and structural changes in myocardium associated with diabetes without coronary artery disease. It is one of the causes of heart failure in diabetic patients besides myocardial ischemia and coronary artery disease. Diabetes mellitus affects the heart by mechanisms including cardiac autonomic dysfunction, inflammation, maladaptive immune response, metabolic disturbance, microvascular impairment, and subcellular abnormalities (Lee and Kim, 2017). Activation of the Renin-Angiotensin-Aldosterone System (RAAS) and oxidative stress are also proposed mechanisms of DCM ( Pappachan *et al.*, 2013; Jia *et al.*, 2016). Hyperglycemia, hyperlipidemia, and inflammation stimulate the production of Reactive Nitrogen Species (RNS) or ROS that cause diabetic complications, including DCM (Inoguchi *et al.*, 2000). Adaptive responses to these metabolic abnormalities result in cardiac dysfunction and heart failure (Pappachan *et al.*, 2013).

DCM can be asymptomatic in first phases, however, in later phases it may appear as overt heart failure. Patients develop symptoms related to advanced heart failure or previous heart failure or both due to lack of pumping ability or impaired venous dumping due to congestion. Angina pectoris, dyspnea, edema in lower extremities, fatigue, hepatomegaly, increased jugular vein pressure, syncope and weakness may appear as symptoms of failing cardiac function (Miki et al., 2013).

Echocardiography can show diastolic dysfunction even in asymptomatic patients and those without any cardiac hypertrophic modifications. Vital aspects of treatment are the minimization of cardiac risk factors, lifestyle changes, regulation of blood glucose levels, and therapy of heart failure in a patient with overt cardiac damage (Ogihara *et al.*, 2009).

Many clinical trials showed that an improvement of glycemic control has been shown to be associated with improved parameters of outcome in diabetic cardiomyopathy. Management of T2DM is associated with improved management options for patients with DCM. Metformin has

been shown to up-regulate cardiomyocyte autophagy that has a role in the prevention of DCM in animal models. Glucagon-like Peptide-1 (GLP-1) is a peptide hormone, secreted by the L-cells of jejunum and ileum of the small intestine that stimulates meal-related endogenous insulin secretion. The use of this drug in obese T2DM patients is associated with improvement in glycemic control and weight loss. This drugs may emerge as a promising treatment option in obese T2DM patients with DCM (Xie *et al.*, 2011).

### 1.1.5 Diagnosis of Diabetes Mellitus

Diabetes can be diagnosed based on blood glucose criteria, either the Fasting Blood Glucose or the 2-hours Blood glucose (2-h BG) value after a 75 g oral glucose tolerance test (OGTT) or HbA1C criteria (Eric, S., 2009). These tests are used for diagnosis of DM in addition to clinical features and family history of the patient. However, it is essential to consider age, anemia or hemoglobinopathies and race in patients while interpreting the results. Table 3 describes the criteria for classification and diagnosis of the diabetic state of the patient (American Diabetes Association, 2017).

Table 3: **Criteria for diagnosis of DM** (American Diabetes Association, 2017).

	<b>Normal</b>	<b>Prediabetes</b>	<b>Diabetes</b>
Random plasma glucose levels	< 11.1 mmol/l	N/A	≥ 11.1 mmol/l
Fasting plasma glucose	< 5.6 mmol/l	5.6-6.9 mmol/l	≥ 7.0 mmol/l
2-hours blood glucose value after a 75g oral glucose tolerance test	< 7.8 mmol/l	7.8-11.0 mmol/l	≥ 11.1 mmol/l
HbA1C	<39 mmol/mol	39-47 mmol/mol	≥48 mmol/mol

### **1.1.6 Management and current treatment for diabetes mellitus.**

Insulin is the main drug for the management of T1DM. Biotechnologically produced insulin is less likely to cause allergic reactions in diabetics. This form of insulin is absorbed more rapidly than animal-derived insulin thus showing its effectiveness in a shorter duration. Table 4 describes various oral drugs for T2DM.

**Table 4: Drugs approved by the FDA for the treatment of Type 2 DM (Navale, 2017).**

<b>Class</b>	<b>Drug</b>	<b>Mechanism of action</b>	<b>Adverse effects</b>
Alpha glucosidase inhibitors	Acarbose, Miglitol	Reduce postprandial blood glucose level by delaying complex carbohydrate absorption.	Gastrointestinal disturbances.
Biguanides	Metformin	Decrease in hepatic gluconeogenesis and increased skeletal muscle glucose uptake through activation of AMPK.	Diarrhea, flatulence, nausea, and vomiting.
Dopamine agonist	Bromocriptine	Reduces insulin resistance mediated by a central mechanism.	Nausea, vomiting, dizziness, headache, diarrhea.
DPP-4 inhibitors	Sitagliptin, Saxagliptin, Linagliptin	Inhibits enzyme dipeptidyl peptidase reducing degradation of incretin hormone and an increase in insulin secretion.	Rare clinically significant side-effects
GLP 1 analogs	Exenatide, Liraglutide	Stimulate insulin secretion, slows gastric emptying, suppresses glucagon levels and leads to weight loss, induce satiety.	Nausea, vomiting, diarrhea
Non sulfonylureas secretagogues	Repaglinide, Nateglinide	Stimulate insulin secretion from $\beta$ cells.	Risk of hypoglycemia
Sulfonylureas: first generation	Glibenclamide Chlorpropamide, Tolazamide,	Stimulate insulin Secretion from $\beta$ cells	Hypoglycemia
Thiazolidinediones	Pioglitazone	Agonist at PPAR- $\gamma$ receptors, improves insulin sensitivity in peripheral tissues.	Edema
Amylin analogs	Pramlintide acetate	Slows gastric emptying promotes satiety.	Nausea
Meglitinides	Repaglinide, Nateglinide	Blocks ATP-dependent potassium channels, stimulates insulin release from pancreatic $\beta$ cells.	Hypoglycemia, associated with rare hepatotoxicity

SGLT2 inhibitors	Dapagliflozin, Canagliflozin, Empagliflozin	Decrease renal glucose reabsorption and thereby enhancing urinary glucose excretion and subsequent reductions in plasma glucose and glycosylated hemoglobin concentration.	Urinary and genital tract infections more common with SGLT2 inhibitors, hypotension
Insulin	Rapid-acting (aspart, glulisine, lispro), long-acting (glargine, detemir)	Binds to the insulin receptor activates glucose transport GLUT4. PIP3 and tyrosine phosphorylated guanine nucleotide exchange proteins, facilitates GLUT4 translocation from the cytosol to the plasma membrane.	Hypoglycemia, weight gain

## **1.2 LIVER DISEASE AND DIABETES.**

### **1.2.1 Structure of liver**

The liver is the largest organ in the human body which is located below the diaphragm in the right upper quadrant of the abdominal cavity. The liver is roughly triangular and consists of two lobes: a larger right lobe and a smaller left lobe. The lobes are separated by the falciform ligament, a band of tissue that keeps it anchored to the diaphragm. An adult's liver weighs around 1.5 kg and extends approximately from the right 5th rib to the lower border of the rib cage. The internal structure of the liver is made of around 100,000 small hexagonal functional units known as lobules (Ramadori et al., 2008). Each lobule consists of a central vein surrounded by 6 hepatic portal veins and 6 hepatic arteries. These blood vessels are connected by many capillary-like tubes called sinusoids. Each sinusoid passes through liver tissue containing 2 main cell types including Kupffer cells and hepatocytes. Kupffer cells are a type of macrophage that capture and break down old, worn out red blood cells passing through the sinusoids whereas; hepatocytes are the working cells of the liver that have a unique capacity to reproduce in response to liver injury. The hepatocytes of the liver are tasked with many of the essential metabolic functions that support the cells of the body (Ramadori *et al.*, 2008).

### **1.2.2 Functions of liver**

The liver plays a major role in the regulation of many of physiological processes in our bodies, which include metabolic, vascular, immunological, storing, secretory and excretory functions. Furthermore, it is involved in detoxification of a variety of drugs and xenobiotics. Also, it plays a key role in the carbohydrate, protein and fat metabolism in the human body (El-bakry *et al.*, 2016). The digestive system breaks down carbohydrates into monosaccharides, which cells use as a primary energy source. Blood which enters the liver through hepatic portal vein is rich in glucose from digested food. Hepatocytes receive this glucose and store it as the macromolecule glycogen, a branched polysaccharide that allows the hepatocytes to collect large amounts of glucose and quickly release glucose between meals. The absorption and release of glucose by the hepatocytes

help to maintain homeostasis and protects the rest of the body from elevation and drops in the blood glucose level (Jensen *et al.*, 2011).

When amino acids enter the liver, they require metabolic processing before they can be used as an energy source. Hepatocytes remove the amine groups of the amino acids and transform them into ammoniac and eventually urea. Urea is less toxic than ammonia and can be excreted in urine as a waste product of metabolism. The remaining parts of the amino acids can be broken down into ATP or transformed into new glucose molecules (Wu, 2009).

The liver synthesizes and excretes bile necessary for digestion and absorption of fats. It also helps in the absorption of vitamin K from the diet. The emulsification of fats by bile turns the large clumps of fat into smaller pieces that have more surface area and are therefore easier for the body to digest (Panjaliya *et al.*, 2013).

Liver stores vitamins such as vitamins A, D, E, K, and B<sub>12</sub>, and the minerals iron and copper in order to provide a continuous supply of these important substances to body tissues.

The liver plays a crucial role in detoxifying substances that are harmful to the body; as blood from the digestive organs passes through the hepatic portal circulation, the hepatocytes of the liver monitor the contents of the blood and remove many potentially toxic substances before they can reach the rest of the body (Panjaliya *et al.*, 2013).

The liver function is an organ immune system through the function of the Kupffer cells that line its sinusoids. Kupffer cells are a type of fixed macrophage that forms part of the mononuclear phagocyte system along with macrophages in the spleen and lymph nodes. Kupffer cells play an important role by trapping parasites, bacteria, fungi, cellular debris and worn-out blood cells (Ramadori *et al.*, 2008).

The liver produces several vital protein components of blood plasma including albumins, fibrinogen, and prothrombin. Albumins are proteins that maintain colloidal osmotic pressure (Yang and Monti., 2017).

### 1.2.3 Clinical signs of liver diseases

**Jaundice:** Jaundice refers to the staining of tissue with bilirubin or bilirubin complexes. It is detectable when the plasma bilirubin exceeds 50 mmol/L (3 mg/dl). The staining is much more

pronounced with conjugated bilirubin (direct), than with unconjugated bilirubin (Ullah *et al.*, 2016).

The usual divisions of jaundice are:

- Hemolytic jaundice (pre hepatic) – increased bilirubin load for the liver cells.
- Hepatocellular jaundice (hepatic or toxic) – defects in conjugation.
- Cholestatic jaundice (obstructive or post-hepatic), including hepatocellular (parenchymal) liver disease and large duct obstruction (Ullah *et al.*, 2016)

### **Hepatic encephalopathy:**

Hepatic encephalopathy (HE) is a term used to describe a spectrum of neuropsychiatric abnormalities in patients with significant liver disease. HE is classified as type A, B, or C based on the underlying mechanism causing the encephalopathy. Type A is caused by acute liver failure, whereas type B is primarily caused by portosystemic shunting of blood in the absence of liver disease and type C is resulting from cirrhosis of the liver with portal hypertension (Bajaj *et al.*, 2009). Lactulose is used to treat or to prevent HE.

In a patient with known or suspected of liver disease, the following clinical features are key to diagnosing overt HE: impaired orientation; agitation; reduced awareness of surroundings succeeded by drowsiness, stupor, and coma; changes in consciousness, rigidities, tremors, and alteration in reflexes (Salgado, M., 2013). Asterixis (flapping tremor) is observed as rhythmic bursts (3-4 per second) of flexion-extension movements of the metacarpophalangeal joints, along with the side-to-side movement of fingers with the active maintenance of posture. These movements are also associated with flexion-extension and radial-ulnar deviation of the wrists (Salgado, M., 2013).

**Edema and emaciation:** In liver failure, there is a low metabolism protein and amino acid which is manifested by tissue wasting and a fall of plasma protein causing edema as a result of lowering the osmotic pressure of the plasma.

**Diarrhea and constipation:** Bile salts have laxative and disinfectant effects, so if they are low or absent in the alimentary tract due to hepatitis, hepatic fibrosis or obstruction of the biliary system, constipation punctuated by attacks of diarrhea may ensue. The feces are pale in color with high-fat contents (steatorrhea).

**Hemorrhagic diathesis:** This occurs in severe diseases of the liver due to a deficiency in prothrombin formation and retardation of the fat-soluble vitamin production (Vit K) (Per H et al., 2013).

**Abdominal pain:** Abdominal pain in liver diseases is caused by enlargement of the liver with increased tension and lesions of the capsule.

#### 1.2.4 Mechanisms of diabetes-associated liver damage

To appreciate the many physical and biochemical alterations that occur in the liver in the presence of diabetes, and to recognize how the liver disease may affect glucose metabolism, understanding of the role of the liver in the regulation of carbohydrate homeostasis is essential. The liver uses glucose and also has the ability to store it as glycogen and synthesize it from non-carbohydrate precursors (gluconeogenesis) (Mohamed, J. et al., 2016). Approximately 70% of persons with T2DM have fatty liver, with necro-inflammation and fibrosis. Evidence suggests that it is not steatosis but the development of lipotoxicity induced-mitochondrial dysfunction and activation of inflammatory pathways that lead to progressive liver damage (Cusi, 2009).

Insulin resistance appears to be a critical contributing factor in the pathogenesis of nonalcoholic fatty liver disease (NAFLD), with obesity as the most common cause of the insulin resistant state. As body fat stores expand with calorie excess and progressive obesity, alterations in lipid metabolism together with inflammation in adipose tissue and ectopic sites of fat deposition lead to insulin resistance predominantly secondary to post-receptor abnormalities in insulin signaling pathways (Andreas and Gerald., 2014). Synthesis of excess triglyceride in the liver is driven by the supply of fatty acids and the accumulation of excess liver fat which is further exacerbated by impaired hepatic fatty acid oxidation secondary to insulin resistance (Bhatt and Smith, 2015).

Insulin resistance most commonly is associated with NAFLD in the context of obesity, and the development and progression of NAFLD usually occur in association with both insulin resistance and a state of ongoing excess calorie intake. There may not only be an increased risk for NAFLD secondary to diabetes, but there also is evidence suggesting that NAFLD conversely may be a risk factor for the development of T2DM (Bhatt and Smith, 2015).

### 1.2.5 Assessment of liver damage

Liver function tests are commonly used in clinical practice to screen for liver complications and disease, monitor the progression of the known disease, and the effects of potentially hepatotoxic drugs. The most common liver function tests include measurement of plasma/serum Alanine Aminotransaminase (ALAT), Aspartate Aminotransaminase (ASAT), Gama-Glutamyltransferase (GGT) and albumin (Harris, 2005).

ALAT and ASAT catalyze the reductive transfer of an amino group from alanine or aspartate, to alpha-ketoglutarate to become glutamate and pyruvate or oxaloacetate, respectively. Damaged hepatocytes release their contents including ALAT and ASAT into the extracellular space. The released enzymes ultimately enter into circulation. ASAT has a broader tissue distribution than ALAT and perturbations in ASAT levels can occur in response to diseases or injuries in multiple tissues including skeletal muscles, brain, liver, and heart. ALAT is primarily found in the liver with lower enzymatic activities in the heart tissue and skeletal muscles (Ozer *et al.*, 2008). GGT is present on the surface of most cell types and highly active in liver, kidney, and pancreas. It has been considered the most specific marker for liver injury. GGT is responsible for the catabolism of extracellular glutathione and may be linked to oxidative stress and chronic inflammation, which are also important pathways for Type 2 diabetes development (Wang *et al* 2016).

Albumins is the most abundant protein in plasma and is synthesized in the liver. Serum albumin is a reliable prognostic indicator for liver disease, nephritic syndrome, malnutrition, and protein-losing enteropathies (Levitt DG and Levitt MD., 2016). The main function of albumins is to maintain colloidal osmotic pressure in plasma. In addition, albumin assists in the transport of different materials, such as vitamins and certain molecules and drugs (Yavuz *et al.*, 2017).

### **1.3 MEDICINAL PLANTS AS SOURCES OF PUTATIVE THERAPEUTIC AGENTS.**

Plants as medicines are mentioned in historic documents dating back many thousands of years. The plants have been used for years to treat diseases (Petrovska, 2012).

It is estimated that 80% of the world population is dependent on traditional medicines for primary healthcare (WHO, 2013). This dependence is significantly due to the fact that plants are considered the only available, affordable and trusted medicine to bring about sustainable solutions to health problems. In spite of considerable progress in modern medicine, there are very few therapeutic agents that can protect the liver from damage and stimulate liver functions (Pathan M et al., 2014). Patients with chronic liver disease are liable to have liver transplantation, which is not only costly but has long-term consequences of immunosuppressive agents, especially hyperlipidemia, hypertension and renal disease (Detlef S and Nezam, 2009). For these reasons, many patients with liver disease use herbal remedies to solve their health problems (Simon P et al., 2010).

In South Africa, there is growing interest in natural plant-based remedies as a source for treatment. Up to 80% of the South African population use traditional medicines to meet their primary health care needs (Street and Prinsloo, 2013).

#### **1.3.1 Medicinal plants used in diabetes management in South Africa.**

A study conducted by Oyedemi *et al.*, (2009) in South Africa especially in the Eastern Cape province, showed that 15 different plant species belonging to thirteen families are used by the traditional healers where *Strychnos henningsii* and *Leonotis leonorus* were the most cited plants in the study. Another study from Limpopo Province South Africa reported that 52 traditional healers interviewed recommended 24 plant species belonging to 20 families for the treatment of diabetes mellitus (Semenya *et al.*, 2012). The most commonly used plants were *Aloe marlothii*, *Helichrysum caespititium*, *Hypoxis iridifolia*, *Mimusops zeyheri*, *Plumeria obtuse* and *Moringa oleifera*.

### 1.3.2 The plant: *Moringa oleifera*

Figure 3: *Moringa oleifera* tree with leaves



### 1.3.2.1 Classification

Plant Name: *Moringa oleifera*

Family: *Moringaceae*

Genus: *Moringa*

Species: *Oleifera L*

### 1.3.2.2 Botanical description: synonyms (Mishra *et al.*, 2011).

Latin : *Moringa oleifera*

Sanskrit : Subhanjana

Hindi : Saguna, Sainjna

Gujarati : Suragavo

Tamil : Morigkai

Telugu : Mulaga, Munaga

Malayalam : Murinna, Sigru

Punjabi : Sainjna, Soanjna

Unani : Sahajan

Ayurvedic : Akshiva, Haritashaaka, Raktaka, Tikshnagandhaa

Arabian : Rawag

French : Moringe à graine ailée, Morungue

Spanish : Ángela, Ben, Moringa

Portuguese : Moringa, Moringueiro

Chinese : La ken

English : Drumstick tree, Horseradish tree, Ben tree

### 1.3.2.3 Geographical source and morphology.

*Moringa oleifera* is the most widely used and Moringaceae family with 13 species. It is a fast-growing, drought-resistant tree that is native to the sub-Himalayan tracts of Afghanistan, Bangladesh, India, and Pakistan; but now dispersed worldwide in the tropics and subtropics. *Moringa* grows best in dry sandy soil, it tolerates poor soil, including coastal areas (Ray *et al.*,

2006). The plant is a domestic tree in South Africa and is commonly planted in Durban and surrounding areas. *Moringa oleifera* is a small, fast-growing evergreen or deciduous tree that usually grows as high as 9 m, with soft and white wood and corky and gummy bark. Roots have the taste of horseradish. Leaves are longitudinally cracked, 30-75 cm long main axis and its branch jointed, glandular at joints, leaflets are glabrous and entire. The leaflets are finely hairy, green and almost hairless on the upper surface, paler and hairless beneath, with red-tinged mid-veins, with entire (not toothed) margins, and are rounded or blunt-pointed at the apex and short-pointed at the base. The twigs are finely hairy and green. Flowers are white, scented in large axillary down panicles, pods are pendulous, ribbed, seeds are 3-angled (Mishra *et al.*, 2011).

#### 1.3.2.4 Bioactive compounds of *Moringa oleifera*.

*Moringa oleifera* has an impressive range of medicinal uses with high nutritional value and medicinal benefits. Different parts of *Moringa* tree contain protein, vitamins A, B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, C, and minerals such as calcium, iron, magnesium, and phosphorus (Gowrishankar *et al.*, 2010). Bioactive nutritional and medicinal compounds are found in its roots, bark, leaves, flowers, fruits and seeds. Phytochemical analysis has shown that the leaves, flowers and pods are good sources of protein, vitamins A, B and C, riboflavin, ascorbic acid, nicotinic acid, folic acid, pyridoxine, beta-carotene, amino acids, iron, calcium, alpha-tocopherol, flavonoids (quercetin, kaempferol) and various phenols (Mbikay, 2012; Chinedu *et al.*, 2015).

The root and bark contain two alkaloids: moringine and moringinine. Other isolated compounds from plants are 4 - (4'-Oacetyl –  $\alpha$  - L- rhamnopyranosyloxy) benzyl, 4 - (-L rhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothiocyanate, and 4 - ( $\alpha$ -L- rhamnopyranosyloxy) benzylglucosinolate4 (Mishra *et al.*, 2011).

#### Pharmacological studies of *Moringa oleifera*.

Pharmacological studies have suggested that MO have antioxidant, antiatherosclerotic, hypolipidaemic, antitumoral, antipyretic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antibacterial and antifungal properties (See table 4 for references). MO leaf is known to be non-toxic to animals at high doses. A study performed by Ekundina *et al.*, (2015) testing the

hepatotoxicity and nephrotoxicity of ethanolic extract of *Moringa oleifera* on liver and kidney at doses of 400, 600 and 800 mg/kg did not show any visible lesions or disease.

**Table 5: Pharmacological actions of *Moringa oleifera*.**

<b>Uses of the plant</b>	<b>References.</b>	<b>Morphological part used</b>
Anti-spasmodic.	(Anwar <i>et al.</i> , 2007)	Aqueous leaf extracts
Anti-atherosclerotic.	(Chumark <i>et al.</i> , 2008)	Aqueous leaf extracts
Anti-bacteria	(Rahman & Sheikh, 2009)	Aqueous leaf extracts
Anti-cancer	(Vasanth <i>et al.</i> , 2014)	Stem bark
Anti-diabetic	(Gupta <i>et al.</i> , 2012; Aja <i>et al.</i> , 2015)	Aqueous leaf extracts
Anti-fungal.	(Kekuda <i>et al.</i> , 2010; Al-Malki & El Rabey, 2015)	Leaf powder
Diuretic.	(Anwar <i>et al.</i> , 2007)	Aqueous leaf extracts
Antihyperglycemic	( Jaiswal <i>et al.</i> , 2009; Edoğa <i>et al.</i> , 2013)	Aqueous leaf extracts
Anti-pyretic.	(Aney <i>et al.</i> , 2009)	Aqueous leaf extracts
Anti-inflammatory.	(Anwar <i>et al.</i> , 2007)	Aqueous leaf extracts
Anti-oxidant.	(Sreelatha & Padma, 2009; Ndhala <i>et al.</i> , 2014)	Aqueous leaf extracts
anti-tumor	(Dawood and Fathy, 2014)	Aqueous leaf extracts
Anti-ulcer.	(Verma <i>et al.</i> , 2012; Charoensin, 2014)	Aqueous leaf extracts
Anti-dyslipidemia.	(Chumark <i>et al.</i> , 2008; Dawood & Fathy, 2014)	Aqueous leaf extracts
hepatoprotective	(Abou-Zeid <i>et al.</i> , 2016; El-bakry <i>et al.</i> , 2016).	Aqueous leaf extracts

#### 1.3.2.5 Hepatoprotective activity of *Moringa oleifera*.

MO has extensively been studied pharmacologically to justify its broad traditional medicinal use, from which it could have hepatoprotective properties. The study by El-bakry et al., (2016) showed that aqueous extracts have protected against CCl<sub>4</sub>-induced acute hepatotoxicity through the restoration of the anti-oxidative defense system and down-regulation of the pro-inflammatory pathway.

A previous study showed that MO has hepatoprotective activity against the hepatotoxic effects of acetaminophen (Abou-zeid *et al.*, 2016). In that study, the protective effects of MO alcoholic leaf extracts against the hepatotoxicity induced by acetaminophen were examined on isolated rat hepatocytes (Abou-zeid *et al.*, 2016). The first set of hepatocytes was exposed to acetaminophen. Other sets of hepatocytes were preincubated with MO extract for 30 minutes before exposure to acetaminophen. Cell viability and enzyme (Lactate Dehydrogenase (LDH), ASAT & ALAT) leakage were assessed. As a result, acetaminophen reduced the hepatocyte viability and increased leakage of LDH, ASAT, and ALAT into incubation medium in a time-dependent manner. However, pretreatment of hepatocytes with MO extract showed increased cell viability, reduced ASAT and ALAT leakage and reduced lipid peroxide levels (Abou-zeid *et al.*, 2016).

MO has shown protection against cadmium-induced hepatotoxicity. Oral administration of cadmium chloride to Wistar albino rats for 28 days showed a significant increase in alkaline phosphatase (ALP), ALAT, ASAT, lipid peroxidation (LPO) and a decrease in superoxide dismutase (SOD), an increase in cadmium accumulation in the liver. Treatment with MO 500 mg/kg significantly decreased the elevated ALAT, ASAT, ALP, LPO levels and an increase of SOD levels, as compared to cadmium chloride treated group (Toppo *et al.*, 2015).

In the study conducted by Saalu *et al.*, (2012), alcohol was administered to induce hepatotoxicity in Wistar rats. Assessment of the liver histology, liver oxidative stress and the activities of liver biomarker enzymes was done in order to investigate the capabilities of polyphenolic antioxidant-rich MO leaf extract to ameliorate liver derangement caused by alcohol. As outcomes, post-treatment with MO leaf extract attenuated liver morphological, dysfunctional and oxidative status changes mediated by alcohol ingestion.

These hepatoprotective properties have been shown to be due to the antioxidant activity of *Moringa oleifera* (phenolic compounds including flavonoids: kaempferol, rhamnetin, quercetin, chlorogenic acid, rutin, apigenin).

It has been reported that oxidative stress plays a main role in the initiation and progression of a variety of liver diseases, and many natural antioxidants have been tried to prevent oxidative stress-mediated liver injury (Xie *et al.*, 2012).

In the absence of reliable therapeutic agents that can protect the liver from damage in modern medicine, remedies from medicinal plants are recommended to stimulate liver functions. Anti-diabetic effects of MO have previously been demonstrated (Aja *et al.*, 2015). Diabetes is associated with hepatotoxicity. This study aims to investigate whether *Moringa oleifera* could mitigate hepatotoxicity associated with diabetes mellitus.

#### **1.4 HYPOTHESIS, AIM AND OBJECTIVES.**

##### **1.4.1 Hypothesis:**

*Moringa oleifera* leaf extracts have hepatoprotective effects in streptozotocin-induced diabetes.

##### **1.4.2 Aim:**

To investigate the hepatoprotective effects of *Moringa oleifera* in streptozotocin-induced diabetes.

##### **1.4.3 Objectives:**

- To determine the effects of MO extracts on glucose intolerance in streptozotocin-induced diabetes.
- To determine the effects of MO extracts on liver function tests in diabetic rats.

## 1.5 MATERIALS AND METHODS

### 1.5.1 Drugs and chemicals.

All chemicals and reagents were purchased from Sigma Aldrich Pty. Ltd., Johannesburg, South Africa. Streptozotocin, citric acid, sodium phosphate, potassium chloride, methanol, phosphate buffer, sodium citrate, sodium chloride, ALAT activity assay kit, ASAT activity assay kit, GGT activity calorimetric assay, insulin ELISA kit, and BCP albumin assay kit were used in the study. Normal saline, insulin, glucometer, and glucometer test strips were purchased from a local pharmacy in Durban-South Africa. Two thousand grams of fresh leaves of fully grown *Moringa oleifera* were locally collected in Durban, South Africa. The harvested leaves and parts of the plant were identified and confirmed by Professor Himansu Baijnath of School of Life Sciences, University of KwaZulu-Natal, South Africa.

### 1.5.2 Animals.

Male Wistar rats (250-300 g) were procured from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa. They were kept in cages and housed in the animal holding facility; and exposed to a 12-hour light, 12-hour darkness cycle in a controlled room (temperature  $24 \pm 1^\circ\text{C}$ , humidity  $55\% \pm 5\%$ ). Animals had free access to standard commercial chow and drinking tap water. They were treated with humane care according to the guidelines of the Animal Research Ethics Committee of the University of KwaZulu- Natal; Ethical clearance number: AREC/038/017M.

### 1.5.3 Preparation of the plant.

The fresh plant samples (leaves) collected were washed under running tap water to remove soil particles and other dirt. The leaves of *Moringa oleifera* were air-dried at room temperature ( $23 \pm 2^\circ\text{C}$ ) for 12 days. The dried leaves were pulverized to fine granules using an electric grinder.

Method of Anwar *et al.*, (2013) was adopted for the preparation of water and aqueous-methanol extracts. For the aqueous extract, 100 g of dry MO leaf powder was weighed and soaked into a 500 ml of distilled water in volumetric flasks. Water was gradually added to this flask and the flask moderately shaken until of uniform consistency was formed. The constituents were then stirred by use of a magnetic stirrer for 48 h and thereafter centrifuged at 3000 revolutions per minute (RPM) for 10 min. The extract was then passed through a Whatman N<sup>o</sup>1 filter paper (24 cm). The residues

were extracted by the same procedure. A mixture of water and methanol (20:80 v/v) was gradually added to this flask and the flask was gently shaken until a slurry of uniform consistency was formed. Supernatant collected was poured into a round-bottomed flask.

Due to the different boiling points of water and methanol, the round-bottomed flask was then attached to a rotary evaporator (EYELA, SB-651, Rik.kikai co.Ltd.Tokyo, Japan) at 40 °C. After running for 8h, methanol was removed. Finally, the extract was weighted and stored at -4°C until use.

#### 1.5.4 Experimental design.

After one week acclimatization period, the rats were randomly assigned into six 6 groups (n=7) as follows: Group A was orally treated daily with 3.0 ml/kg/body weight (BW) of distilled water; group C was similarly treated with MO (500 mg/kg/BW) daily. Groups B, D, E, and F were rendered diabetic by a single intraperitoneal injection of STZ (45 mg/kg/BW in 0.1M citrate buffer, pH4.5). Diabetes was confirmed 3 days later. Additionally, group D was treated with subcutaneous insulin (2 U/kg/BW, bid) while groups E and F were orally treated daily with MO 250 mg/kg/BW and 500 mg/kg/BW, respectively. Administration of STZ was done on day 1. Administration of MO/insulin started three days later when hyperglycemia was established.

Table 6: **Animal treatment protocol**

<b>Treatment</b>	<b>Distilled Water</b>	<b>MO</b>	<b>MO</b>	<b>STZ</b>	<b>Insulin</b>
<b>Groups</b>	ml/kg BW	mg/kg BW	mg/kg BW	mg/kg BW	IU/kg BW
A	3.0	Nil	Nil	Nil	Nil
B	Nil	Nil	Nil	45	Nil
C	Nil	Nil	500	Nil	Nil
D	Nil	Nil	Nil	45	2.0
E	Nil	250	Nil	45	Nil
F	Nil	Nil	500	45	Nil

Only 500 mg/kg BW of MO was used to treat non-diabetic rats. This dose displayed the maximum of effects that could have been displayed when using 250 mg/kg BW.

#### 1.5.5 Induction of diabetes

##### **Preparation of citrate buffer and STZ solutions.**

**Citrate buffer** (0.1 M, pH 4.4): To prepare 0.1 M citrate buffer (pH 4.4), 1.92 g of citric acid crystals (MW of 192.12, Sigma Aldrich, Johannesburg, South Africa) and 2.94 g of sodium citrate dihydrate (M.W. of 294.10, BDH Merck Ltd., Poole, England) were separately dissolved in 100 ml of distilled water. Then, these two solutions were mixed together to adjust the pH 4.4 by using a pH meter (Lasec SA (Pty) Ltd).

**Normal saline (0.9% NaCl) solution:** A 0.9 g of sodium chloride (M.W. of 58.44, Merck Chemicals (PTY) LTD) was dissolved in 100 ml of distilled water.

**Streptozotocin solution:** 333 mg of STZ was dissolved in 26 ml of citrate buffer (12 mg/ml) and administered at 45 mg/kg BW to the rats.

Diabetes was induced in animals of groups B, D, E and F by single intraperitoneal injection of STZ (45.0 mg/kg body weight) dissolved in 0.2 mL of 0.1 M citrate buffer, pH 4.5. Three days after STZ administration, development of hyperglycemia was confirmed by tail picking to analyze blood glucose levels using glucometer machine with trips. Rats with fasting blood glucose (FBG) more than 6.0 mmol/L were included in the study.

Body weights were measured daily. Body weights were measured daily. Seven rats were housed per cage. Graduated bottles of water were placed on animal cages. Calculation of average consumption per cage was expressed by kilogram of body weight.

#### 1.5.6 Glucose Tolerance Tests (GTT).

Glucose tolerance tests (GTT) were done on day 47 of the animal treatment. After an overnight fast for 8 hours, rats in all groups were intraperitoneally dosed with a D-glucose solution (3.0 g/kg BW) in 0.9% normal saline. This solution was prepared by dissolving 45 g of D-glucose anhydrous

in 60 ml distilled water (0.75 g/ml). Blood glucose concentrations were measured by tail pricking at 0, 30, 60, 90, and 120 minutes, using glucometer (OneTouch select®; Lifescan Inc., Milpitas, California, USA).

The Area under the Curve (AUC) was calculated from blood glucose-time curves and presented as AUC units (mg/dl/min) (Scheff *et al.*, 2012). On day 54 of treatment, animals were sacrificed by halothane overdose. Blood was collected by cardiac puncture in heparinized tubes then separated into plasma and stored at -80°C for further biochemical analysis. Livers were excised, snap-frozen in liquid nitrogen and similarly stored for histological analysis.

### 1.5.7 Plasma Insulin concentrations and calculation of HOMA-IR score.

An ultrasensitive rat insulin ELISA kit was used to determine the concentration of insulin as per manufacturer's instructions. Calibrators (25 µl), and the plasma samples in triplicate were pipetted into the 96 well microtiter-plate. One hundred (100) µl of the enzyme conjugate solution was then added into each well. This was then followed by incubation on a plate shaker at 700-900 rpm for 2 hours at room temperature. The plate was then washed 6 times with 700 µl of washing buffer solution per well. After the final wash, the plate was inverted and tapped on dry absorbent paper in order to remove excess washing buffer. Two hundred (200) µl of substrate IMP was then added into each well. The plate was then incubated at room temperature for 15 minutes. Fifty (50) µl of the stop solution was then added and then the microtiter plate was placed on the plate shaker for 5 seconds in order to ensure a maximum reaction. The microtiter plate was then measured at 450 nm.

Insulin resistance was calculated by using the Homeostasis Model Assessment of Insulin resistance (HOMA-IR) equation (Gayoso-diz *et al.*, 2013):

Insulin Resistance = [Fasting Plasma Insulin (µU/ml) x Fasting plasma glucose (mmol/L)]/22.5].

$$IR = \frac{(FPI \times FPG)}{22,5}$$

Where IR is Insulin Resistance, FPI is the Fasting Plasma Insulin and FPG is the Fasting Plasma Glucose.

### 1.5.8 Liver function tests

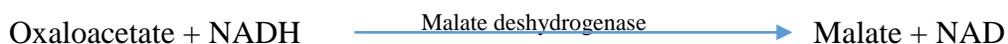
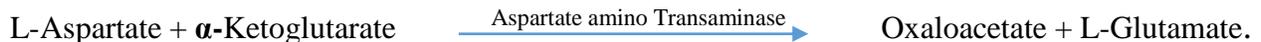
- **Alanine amino transaminase (ALAT):**

Alanine aminotransaminase (ALAT) is an enzyme involved in the metabolism of the amino acid alanine. ALAT works in a number of tissues, but its highest concentrations are in the liver. An increase of serum ALAT levels in blood circulation has been used as an indicator for liver injury (Arun *et al.*, 2012). ALAT catalyzes the transfer of amino groups from alanine to  $\alpha$ -ketoglutarate which generates pyruvate and glutamate. Lactate dehydrogenase reduces lactate to pyruvate and oxidizes NADH to NAD. The rate of decrease in absorbance of the reaction mixture at 340 nm, due to the oxidation of NADH is directly proportional to the ALAT activity.



- **Aspartate amino transaminase (ASAT):**

Aspartate amino transaminase (ASAT) formerly called serum glutamate-oxaloacetate transaminase (SGOT), plays a role in catalyzing the transfer of amino groups from aspartic acid to  $\alpha$ -ketoglutarate which generates oxaloacetate and glutamate. Malate dehydrogenase reduces oxaloacetate to malate and oxidizes NADH to NAD. The rate of decrease in absorbance of the reaction mixture at 340 nm, due to the oxidation of NADH is directly proportional to the ALAT activity.



- **Gamma Glutamyl transferase (GGT).**

Gamma-Glutamyltransferase is membrane-bound protein that catalyze the transfer of Gamma-glutamyl moiety from Gamma-glutamyl peptides to other peptides such as glutathione and to L-amino acids. Serum GGT levels can be elevated in many pathophysiological conditions such as cardiovascular disease, metabolic syndrome, and chronic liver disease. Elevated serum levels of GGT can also be indicative of oxidative stress. GGT is the most specific marker for liver injury.

Amino acid + Glutathione  $\xrightarrow{\text{Gamma-glutamyl transferase}}$  Glutamyl amino acid + Cysteinylglycine.

- **Albumin**

Albumin is produced in the liver and makes up 60% of plasma protein. Albumin plays an important physiological role in maintaining the osmotic pressure of plasma. Also, albumin binds and transports drugs, vitamins, and other molecules. Albumin is measured as an endpoint reaction with albumin binding to Bromocresol green (BCG), which is an anionic dye, in an acidic environment. The increase in absorbance at 620 nm of the green colored product is relative to the albumin concentrations in the sample (Moman and Bhimji, S., 2018 ).

### 1.5.9 Histological analysis.

A standard laboratory protocol for paraffin embedding was followed in processing the formalin-preserved liver tissues. Liver specimens excised from experimental animals were washed and fixed in 10% buffered formalin. Tissues were processed by embedding in paraffin and sectioned (4  $\mu$ m thickness). Thereafter, *p*-xylene was used to deparaffinize the slides which were then rehydrated in ethanol concentration gradient (100%, 80%, 70%, and 50%) and rinsed with tap water (Essawy *et al.*, 2012). After, the hematoxylin and eosin were used to stain the slides for 5 minutes which were rinsed with water. As a final point, the slides were mounted in DPX, cover-slipped and viewed with Leica slide scanner (SCN 4000, Leica Biosystems Germany) (Yahya *et al.*, 2013).

#### 1.5.10 Statistical analysis

Data were expressed as mean  $\pm$  standard deviation. One-way ANOVA or student t-tests were used to determine statistical significance for normal distribution data, while KRUSKAL-WALLIS H or Mann-WHITNEY U (Nonparametric tests) were used for non-normal distribution. GraphPad Prism Software Version 5.0. (GraphPad Prism® Software, Inc. San Diego, CA, USA) was used to compare the statistical difference between control means and treatment groups. Values of  $p < 0.05$  were considered statistically significant.

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## CHAPTER 2: RESULTS.

After presenting the literature review, methods, aim and objectives of the study in chapter one, the general findings, discussion and results of the study were further reported in chapter two. A paper was presented in the form of a manuscript titled “Hepatoprotective effects of *Moringa oleifera* leaf extracts in streptozotocin-induced diabetes”. This manuscript is submitted to Journal of Functional Foods for publication.

**Hepatoprotective effects of *Moringa oleifera* Lam (Moringaceae) leaf extracts in streptozotocin-induced diabetes.**

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## **Abstract**

Effects of methanolic leaf extracts of *Moringa oleifera* (MO) on hepatic injury in streptozotocin (STZ)-induced diabetes were investigated.

Male Wistar rats were divided into 6 groups (n=7). Animals in group A were orally treated daily with 3.0 ml/kg/body weight (BW) of distilled water, while groups C, E and F were similarly treated with 500, 250 and 500 mg/kg/BW, respectively with MO. Groups B, D, E and F were rendered diabetic by single intraperitoneal injections of 45 mg/kg/BW of STZ. Additionally, group D was treated with subcutaneous insulin (2.0 U/kg/BW, bid).

Diabetic animals exhibited significant weight loss, polydipsia, impaired glucose tolerance, fasting hypoinsulinemia and impaired liver function tests compared to controls. Treatment with MO methanolic leaf extracts significantly improved weight loss, polydipsia, glucose tolerance and also liver function tests in diabetic animals.

MO has dose-dependent antidiabetic and hepatoprotective effects.

## **Keywords**

*Moringa oleifera*, diabetes, streptozotocin, liver.

## **Abbreviations**

ADA, American diabetes association; ANOVA, one way analysis of variance; ALAT, Alanine Aminotransaminase; ASAT, Aspartate Aminotransaminase; AUC, areas under the curve; BW, body weight; FBG, fasting blood glucose; FPI, fasting plasma insulin; GC-MS chromatography-mass spectrometry; GGT, gamma-glutamyl aminotransaminase; MO, *Moringa oleifera*; OGGT, oral glucose tolerance tests; STZ, streptozotocin.

## 1.0 Introduction

Diabetes mellitus is associated with increased morbidity and mortality globally and 425 million adults aged between 20 and 79 years were diagnosed with diabetes in 2017 (IDF, 2018; Ogurtsova *et al.*, 2017). Due to defects in insulin secretion, insulin action, or both persistent hyperglycemia affects carbohydrate, lipid and protein metabolism (ADA, 2017). Long term complications include hepatic injury characterized by cirrhosis, inflammation, apoptosis, and microvascular and macrovascular aberrations (ADA 2009 ).

Liver function tests are commonly used in clinical practice to diagnose and monitor liver diseases. Liver enzymes such as Alanine Aminotransferase (ALAT; EC 2.6.1.2), Aspartate Aminotransferase (ASAT; EC 2.6.1.1), Gama-glutamylaminotransferase (GGT; EC 2.3.2.2) and albumin are routinely used as biomarkers for hepatocyte integrity (Harris, 2005). GGT is more specific to liver injury, is responsible for the catabolism of extracellular glutathione and is elevated in conditions of increased oxidative stress and inflammation such as diabetes (Wang et al, 2016). ALAT is specific for hepatocyte necrosis and although ASAT levels may be elevated in other conditions such as heart failure or myocardial infarction, increased levels are also indicative of liver disease. The liver synthesizes albumin, and hepatocellular inflammation and increased oxidative stress which are common conditions in persistent hyperglycemia is known to cause hypoalbuminemia (Levitt & Levitt, 2016).

It has been reported that 80% of the world's population use traditional medicines in primary healthcare due to their abundance, affordability, safety, and efficacy (WHO Traditional Medicine & Strategy, 2014). In South Africa for example, 80% of the population uses some sort of traditional medicines to meet their primary health care needs (van Wyk, 2008). One of such medicinal plants is MO which is commonly called "drumstick tree" in English, "Zogale" and "George," in Hausa and Igala languages of Nigeria, respectively (Atawodi et al., 2010). MO was first discovered in India, Asia and some parts of Western Africa in countries such as Ghana and Senegal (Booth, 1988). In the Limpopo province of South Africa, MO is grown by small scale farmers and the leaves processed into a powder that is sold commercially in local markets (Mabapa et al, 2017). The leaves can be harvested as early as 3 months post-planting for domestic use as a food source or dried to concentrate nutrients and facilitate preservation, then ground by traditional methods into fine powder that acts as feed source for both humans and domestic animals (Mabapa et al,

2017; Moyo et al., 2011; Maroyi, 2006; Gadziray et al., 2013). MO has 13 drought resistant subspecies that are fast growing (up to 6-7 m/year) in different parts of the world (Mabapa et al., 2017; Odee, 1998). In the province of KwaZulu-Natal, as in the rest of the country, traditional healers use different morphological parts of the plant to treat infectious, chronic and acute degenerative ailments.

It has since emerged that MO has human and animal nutritive value owing to the high contents of proteins, flavonoids, vitamins and minerals such as calcium, iron, magnesium, and phosphorus (Gowrishankar *et al.*, 2010; Chinedu et al., 2014; Mishra et al., 2011). Furthermore, MO extracts have been credited with pharmacological effects such as antioxidant (Charoensin, 2014; Ndhala *et al.*, 2014), antiatherosclerotic and hypolipidemic (Chumark *et al.*, 2008; Yassa & Tohamy, 2014), antineoplastic (Charoensin, 2014; Yassa & Tohamy, 2014; Vasanth et al., 2014), antipyretic (Aney *et al.*, 2009), anti-inflammatory, antispasmodic and diuretic (Anwar *et al.*, 2007), antibacterial (Ndhala *et al.*, 2014), antifungal (Kekuda *et al.*, 2010), anti-hyperglycemic (Edoga *et al.*, 2013) and hepatoprotective effects (Abou-Zeid *et al.*, 2016; El-bakry *et al.*, 2016).

Glucotoxicity associated with diabetes is known to cause inflammation and oxidative stress which interfere with lipid and glucose metabolism (Mota et al., 2016). A Japanese Society of Diabetes study previously reported that the leading cause of death in patients with diabetes mellitus was a chronic liver disease (Shima et al., 2013). It is envisaged that with the high prevalence of type 2 diabetes globally (Ogurtsova et al., 2017), a significant number of people could be afflicted with hepatic dysfunctions secondary to chronic hyperglycemia.

To the best of our knowledge, the effects of MO leaf extracts on hyperglycemia-related hepatotoxicity have not previously been studied. It is envisaged in this study that besides its known antihyperglycemic effects, MO leaf extracts could mitigate the development of hyperglycemia-induced hepatic injury in experimental animals.

## 2.0 Materials and Methods

### 2.1 Reagents

All chemicals and reagents unless otherwise stated were purchased from Sigma Aldrich Pty. Ltd., Johannesburg, South Africa. Diagnostic kits for insulin, ALAT, ASAT, GGT and albumin, streptozotocin, citric acid, sodium phosphate, potassium chloride, methanol, phosphate buffer, sodium citrate, sodium chloride were used in the study. Normal saline, insulin, glucometer, and glucometer test strips were purchased from a local pharmacy in Durban, South Africa.

### 2.2 Plant material and preparation of methanol extracts.

Two thousand grams fresh leaves of MO were collected in Durban, South Africa and identified by an expert Botanist at the herbarium in the School of Life Sciences, University of KwaZulu-Natal. The leaves were then washed with running tap water to remove soil particles and other dirt, and air-dried under room temperature ( $30 \pm 2^\circ \text{C}$ ) for 12 days. The dried leaves were pulverized to fine granules using an electric grinder. Water and aqueous-methanol extracts were prepared according to the modified methods of Makkar et al, (1996) and Sultana and Anwar, (2007). Briefly, 100 g of dry MO leaf powder was weighed and soaked in 500 ml of distilled water in a volumetric flask. Water was gradually added to the flask which was then shaken with a magnetic stirrer for 48 hours until a slurry of uniform consistency was formed and then centrifuged at 3000 rpm for 10 min. The extract was then filtered by Whatman filter paper (24 cm). The residue was re-extracted and a mixture of water and methanol (20:80 v/v) gradually added to this flask with gentle shaking until a slurry of uniform consistency was again formed. Supernatants collected were dispensed into round-bottomed flasks which were then rotary evaporated at  $40^\circ \text{C}$ . Finally, the dried extract residues were weighed and stored at  $-4^\circ \text{C}$  until use.

### 2.3 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

A PerkinEmler<sup>®</sup> Gas Chromatography (Clarus<sup>®</sup> 580) equipped with MSD mass spectrometer (Clarus<sup>®</sup> SQ8S) instrument with built-in autosampler was used carry out GC-MS analysis. Analytical parameters were set as Elite-5MS (30 m x 0.25 mm id x 0.25  $\mu\text{m}$ ) column; oven temperature 37 to  $320^\circ \text{C}$  at a rate of  $18\text{-}25^\circ \text{C}/\text{min}$  and held for 0.5 and 1.85 mins at 18 and  $320^\circ \text{C}$ , respectively; injector temperature was  $250^\circ \text{C}$ ; MS Ion Source temperature was  $280^\circ \text{C}$  with full scan and solvent delay of 0–2.30 min; ms scan Range was m/z 35 – 500 in 0.10 sec ; carrier gas was Heat split flow of 20 ml/min; sample volume was 1.0  $\mu\text{l}$ .

#### 2.4 Animals treatment.

Male Wistar rats (250-300 g) were procured from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal. They were kept in cages and housed in the animal holding room facility and exposed to a 12-hour light/dark cycle in a controlled room (temperature  $24\pm 1^{\circ}\text{C}$ , humidity  $55\pm 5\%$ ). The animals had free access to standard commercial chow and tap drinking water. They were treated with humane care according to the guidelines of the Animal Research Ethics Committee of the University of KwaZulu-Natal; Ethical clearance number: AREC/038/017M.

#### 2.5 Experimental design and protocol

After one week of acclimatization, the rats were randomly divided into 6 groups ( $n=7$ ). Rats in group A served as normal control and were daily orally dosed with 3.0 ml/kg body weight (BW) of distilled water. Groups B, D, E, and F were rendered diabetic by a single intraperitoneal injection of 45 mg/kg BW of streptozotocin (STZ) in 0.2 ml of 0.1 M citrate buffer pH 4.5, after an overnight fast. Rats in groups C and F were orally treated daily with 500 mg/kg BW of MO. Group E was orally treated daily with 250 mg/kg BW of MO. Additionally, group D animals were treated with subcutaneous injections of insulin (2.0 IU bid), (Table 1). Administration of STZ was done on day 1. Administration of MO/insulin started three days later when hyperglycemia was established.

**Table 1.** Animal treatment protocol. The animals were orally treated daily with distilled water, MO methanolic leaf extracts, regular insulin and a single intraperitoneal injection of STZ in citrate buffer, respectively. (BW = Body Weight).

<b>Treatment</b>	<b>Distilled Water</b>	<b>MO</b>	<b>MO</b>	<b>STZ</b>	<b>Insulin</b>
<b>Groups</b>	ml/kg BW	mg/kg BW	mg/kg BW	mg/kg BW	IU/kg BW
A	3.0	Nil	Nil	Nil	Nil
B	Nil	Nil	Nil	45	Nil
C	Nil	Nil	500	Nil	Nil
D	Nil	Nil	Nil	45	2.0
E	Nil	250	Nil	45	Nil
F	Nil	Nil	500	45	Nil

Three days after STZ administration, development of diabetes was confirmed by tail pricking to analyze blood glucose levels using hand-held glucometer with trips. Rats with fasting blood glucose (FBG) more than 6.0 mmol/L were included in the study. Administration of MO/insulin starts immediately after confirmation of diabetes and ends 24 hours before the sacrifice of animals.

Animals were treated consecutively for 53 days, they were sacrificed 24 hours after the last treatment or dose of MO/insulin. GTT was done by intraperitoneal injection of 3.0 g/kg BW of D-glucose in 0.9% normal saline on day 47 after an overnight fast. Blood glucose concentrations were similarly measured at 0, 30, 60, 90, and 120 minutes. Areas under the Curve (AUC) were calculated from blood glucose-time plots and expressed as AUC units (millimoles /liter X minutes). The animals were then sacrificed by halothane overdose after 54 days and blood drawn by cardiac puncture in heparinized tubes and separated into plasma that was kept at -80°C for further biochemical analysis. Livers were excised, divided into equal halves which were either snap-frozen in liquid nitrogen for further biochemical analysis or kept in phosphate buffered-formalin for histological analysis.

#### 2.5.1 Liver function tests

Liver function biomarkers ALAT, ASAT, GGT, and Albumin were measured by commercial kits as per the manufacturer's instructions.

#### 2.5.2 Fasting plasma insulin

Plasma insulin levels were analyzed by ultrasensitive rat insulin enzyme-linked immunoassay kit (DRG Instruments GmbH., Marburg, Germany) as per the manufacturer's instructions.

#### 2.5.3 Histological analysis

Standard laboratory protocol for paraffin embedding was followed in the processing of the formalin-preserved liver tissues. The livers were washed and fixed in 10% phosphate-buffered formalin. The tissues were then embedded in paraffin and sectioned into slices of 4 µm thickness. Thereafter, *p*-xylene was used to deparaffinize the slides which were then rehydrated in ethanol concentration gradient (100%, 80%, 70%, and 50%) and rinsed with tap water (Essawy *et al*, 2012). Hematoxylin and eosin were used to stain the slides for 5 minutes then rinsed with water.

The slides were then mounted in Distyrene, Plasticizer and Xylene (DPX) resin, cover-slipped and viewed with Leica slide scanner (SCN 4000, Leicabiosystems Germany) (Yahya *et al.*, 2013).

## 2.6 Statistical analysis

Data were expressed as mean  $\pm$  standard deviation. One-way ANOVA or Student's t-tests were used to determine statistical significance for normal distribution data, while KRUSKAL-WALLIS H or Mann-WHITNEY U (Nonparametric tests) were used for non-normal distribution. GraphPad Prism Software Version 5.0. (GraphPad Prism<sup>®</sup> Software, Inc. San Diego, CA, USA) was used to compare the statistical differences between treatment groups. Values of  $P < 0.05$  were considered statistically significant.

## 3.0 Results

### 3.1 GC-MS analysis

Potentially bioactive constituents of the MO leaf extracts were separated and identified by GC-MS with an in-built automated mass spectral software that directly compared the peaks and retention times with those of standard compounds in the library following characteristic fragmentation patterns of mass spectra of the identified compounds.

Identified compounds included hydrocarbons, acids, esters, alcohols, and aromatics. Most prominent peaks were identified as acetic acid, octyl ester, 2-pyrrolidinone, furan-2-carboxylic acid, malic acid, glycine, benzene acetic acid, 4-hydroxy-, ethyl ester, D-galactopyranoside, D-glucopyranoside, 9H-pyrido[3,4-b]indole, cyclopentane, 1-isopropylidene-2-trimethylsilyl, cis-7,10,13-Hexadecatrienal, benzyl- $\beta$ -D-glucoside, octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl, 2-(2-Bromoethyl)-3-methyl-oxirane (Table 2).

**Table 2**

Representative compounds identified by MS scans from integrated chromatogram peaks. The most prominent peaks are highlighted in bold. R.T (Retention Time), A/H (Area/Height ratio).

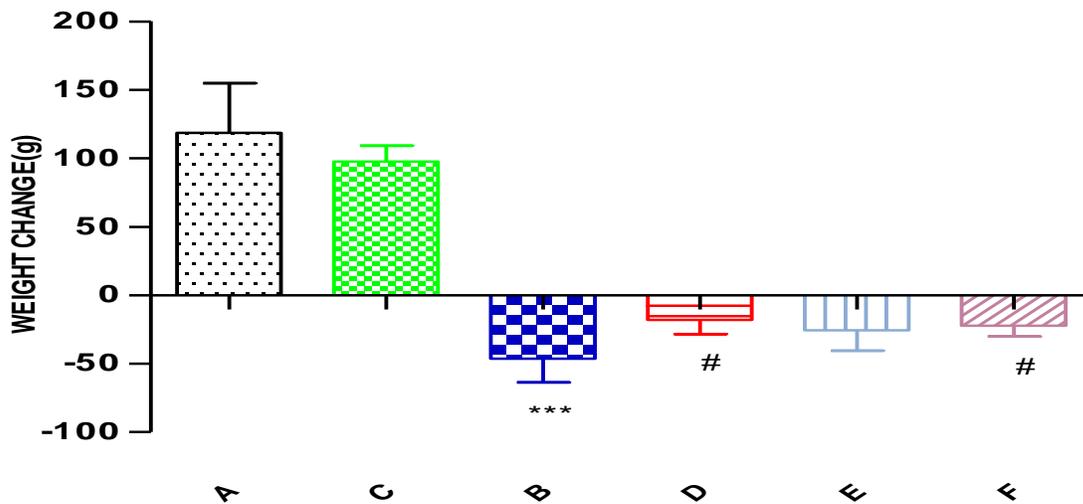
Peak#	R. Time	Area	Area%	Height	Height%	A/H	Name
3	3.028	231622	0.77	2639841	4.47	0.85	1-Propanamine, 2-methyl-N-(2-methylpropylidene)
6	3.661	746671	0.94	1952843	3.30	1.41	Butyrolactone
9	3.802	5289671	1.82	2484839	4.20	2.13	N-(2-Methylbutylidene)isobutylami
10	3.877	1443668	0.50	740235	1.25	1.95	N-methylene-n-octylimine
13	4.418	1961438	0.67	716172	1.21	2.74	Pyrazine, 2-ethyl-3-methyl-
14	4.488	1035382	0.36	250872	0.42	4.13	2-Hexenoic acid
<b>15</b>	<b>4.606</b>	<b>2798953</b>	<b>0.96</b>	<b>593850</b>	<b>1.00</b>	<b>4.71</b>	<b>Acetic acid, octyl ester</b>
16	4.670	5652995	1.94	2934702	4.96	1.93	1-Butanamine, 2-methyl-N-(2-methylbutylidene)
17	4.759	4000179	1.37	1448581	2.45	2.76	1-Butanamine, 3-methyl-N-(3-methylbutylidene)-
18	4.802	5501102	1.89	1410588	2.39	3.90	2-Propenoic acid, 2-methyl-, 1-methylethyl ester
<b>20</b>	<b>5.095</b>	<b>5248693</b>	<b>1.80</b>	<b>1076697</b>	<b>1.82</b>	<b>4.87</b>	<b>2-Pyrrolidinone</b>
21	5.156	1785509	0.61	785709	1.33	2.27	[1,3]Diazepan-2,4-dione
25	5.704	7071155	2.43	3259889	5.51	2.17	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6
26	5.834	2443005	0.84	849869	1.44	2.87	Ethyl hydrogen succinate
<b>28</b>	<b>6.005</b>	<b>1778374</b>	<b>0.61</b>	<b>422370</b>	<b>0.71</b>	<b>4.21</b>	<b>Furan-2-carboxylic acid [2,2,2-trifluoro-1-(2-o</b>
30	6.274	3321809	1.14	944318	1.60	3.52	Benzofuran, 2,3-dihydro-
32	6.502	1166600	0.40	338981	0.57	3.44	Benzeneacetic acid
<b>33</b>	<b>6.763</b>	<b>2078329</b>	<b>0.71</b>	<b>479400</b>	<b>0.81</b>	<b>4.34</b>	<b>Malic Acid</b>
35	7.034	1631466	0.56	565238	0.96	2.89	2-Methoxy-4-vinylphenol
<b>40</b>	<b>7.940</b>	<b>1095895</b>	<b>0.38</b>	<b>167187</b>	<b>0.28</b>	<b>6.55</b>	<b>Glycine, N-(trifluoroacetyl)-, 1-methylbutyl ester</b>
42	8.282	2611355	0.90	874437	1.48	2.99	Benzeneacetonitrile, 4-hydroxy-

<b>45</b>	<b>8.762</b>	<b>5525823</b>	<b>1.90</b>	<b>696614</b>	<b>1.18</b>	<b>7.93</b>	<b>Benzeneacetic acid, 4-hydroxy-, ethyl ester</b>
46	8.803	1382837	0.47	554126	0.94	2.50	1H-Pyrrole, 2-phenyl-
48	9.003	1370724	0.47	890099	1.51	1.54	1H-Pyrrole, 2-(2,4,6-cycloheptatrienyl)-
51	9.325	1640494	0.56	617500	1.04	2.66	Megastigmatrienone
52	9.388	1123407	0.39	410069	0.69	2.74	Ethyl 9-decenoate
<b>53</b>	<b>9.593</b>	<b>21509411</b>	<b>7.38</b>	<b>2913249</b>	<b>4.93</b>	<b>7.38</b>	<b>alpha.-D-Galactopyranoside, methyl</b>
<b>54</b>	<b>9.720</b>	<b>47517811</b>	<b>16.31</b>	<b>3338788</b>	<b>5.65</b>	<b>14.23</b>	<b>Ethyl.alpha.-d-glucoopyranoside</b>
<b>55</b>	<b>9.943</b>	<b>15564596</b>	<b>5.34</b>	<b>1501540</b>	<b>2.54</b>	<b>10.37</b>	<b>Ethyl .alpha.-d-glucoopyranoside</b>
<b>60</b>	<b>10.982</b>	<b>18914584</b>	<b>6.49</b>	<b>1645304</b>	<b>2.78</b>	<b>11.50</b>	<b>Octyl-.beta.-D-glucoopyranoside</b>
<b>64</b>	<b>11.727</b>	<b>13643205</b>	<b>4.68</b>	<b>1876552</b>	<b>3.17</b>	<b>7.27</b>	<b>9H-Pyrido[3,4-b]indole</b>
<b>65</b>	<b>11.807</b>	<b>28277500</b>	<b>9.70</b>	<b>2209710</b>	<b>3.74</b>	<b>12.80</b>	<b>Cyclopentane, 1-isopropylidene-2-trimethylsilyl</b>
<b>66</b>	<b>12.223</b>	<b>1560703</b>	<b>0.54</b>	<b>258284</b>	<b>0.44</b>	<b>6.04</b>	<b>cis,cis,cis-7,10,13-Hexadecatrienal</b>
<b>68</b>	<b>12.985</b>	<b>3087096</b>	<b>1.06</b>	<b>490810</b>	<b>0.83</b>	<b>6.29</b>	<b>Benzyl .beta.-d-glucoside</b>
<b>69</b>	<b>13.431</b>	<b>1589967</b>	<b>0.55</b>	<b>263882</b>	<b>0.45</b>	<b>6.03</b>	<b>Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl</b>
70	14.018	2609788	0.90	713437	1.21	3.66	2-Dimethyl(trimethylsilylmethyl)silyloxy methyltetrahydrofuran
71	14.203	1251510	0.43	288339	0.49	4.34	3,5-O-Benzylidene-1-deoxy-d-gluco-d-guloheptitol
72	14.302	1402047	0.48	427768	0.72	3.28	beta.-l-Rhamnofuranoside, 5-O-acetylthio-octy
<b>75</b>	<b>15.433</b>	<b>1294605</b>	<b>0.44</b>	<b>101719</b>	<b>0.17</b>	<b>12.73</b>	<b>2-(2-Bromoethyl)-3-methyl-oxirane</b>

### 3.2.0 Evidence of diabetes

#### 3.2.1 Animal weights

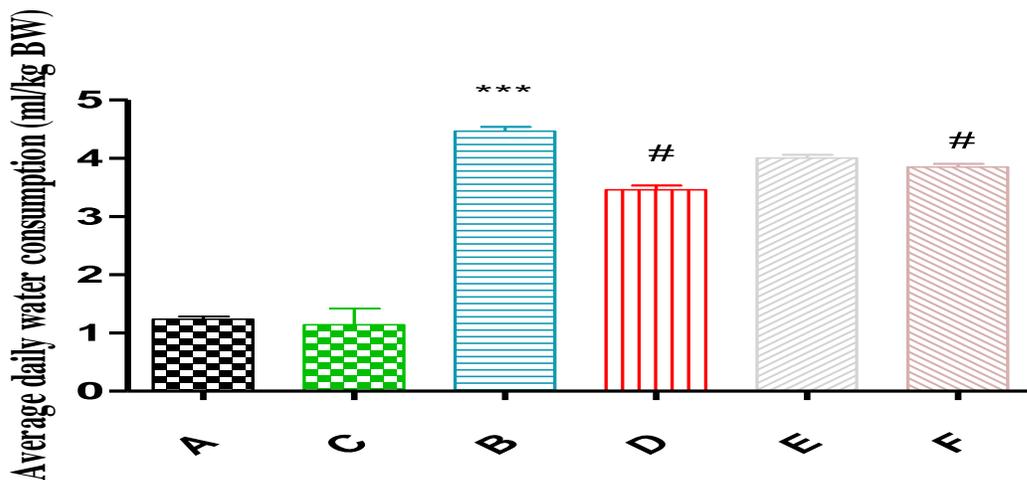
Diabetic rats exhibited significantly ( $p < 0.05$ ) reduced weight gain compared to controls. Treatment with either MO (500 mg/kg BW) or insulin significantly ( $p < 0.05$ ) reduced weight loss in diabetic rats compared to untreated diabetic group (Fig 1). There were no significant differences between MO treated non-diabetic and control rats or between MO-treated (250 mg/kg BW) and non-treated diabetic rats. There was a weight loss in the diabetic groups (Groups B, D, E, and F).



**Fig. 1.** Change in animal weights between day 0 and day 54 of treatment. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated diabetic; E= MO (250 mg/kg BW)-treated, diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*,#  $p < 0.05$  compared to control (A) and non-treated diabetic group (B), respectively)

### 3.2.2 Water consumption

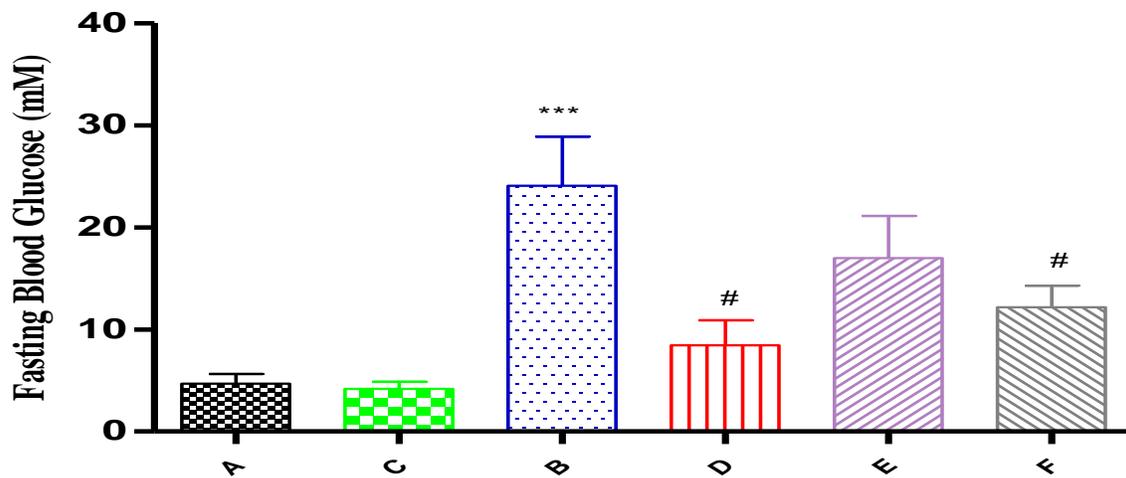
Average water intake was significantly ( $p < 0.05$ ) higher in non-treated diabetic animals compared to controls. Treatment with either MO (500 mg/kg BW) or insulin (2.0 IU/kg BW, bid) significantly ( $p < 0.05$ ) reduced water intake in diabetic rats compared to non-treated diabetic rats (Fig. 2). There were no significant differences between MO treated non-diabetic and control rats or between MO-treated (250 mg/kg BW) and non-treated diabetic rats.



**Fig. 2.** Average daily water consumption in treatment groups. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated, diabetic; E= MO (250 mg/kg BW)-treated, diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*,#  $p < 0.05$  compared to control (A) and non-treated diabetic group (B), respectively)

### 3. 2.3 Fasting blood glucose

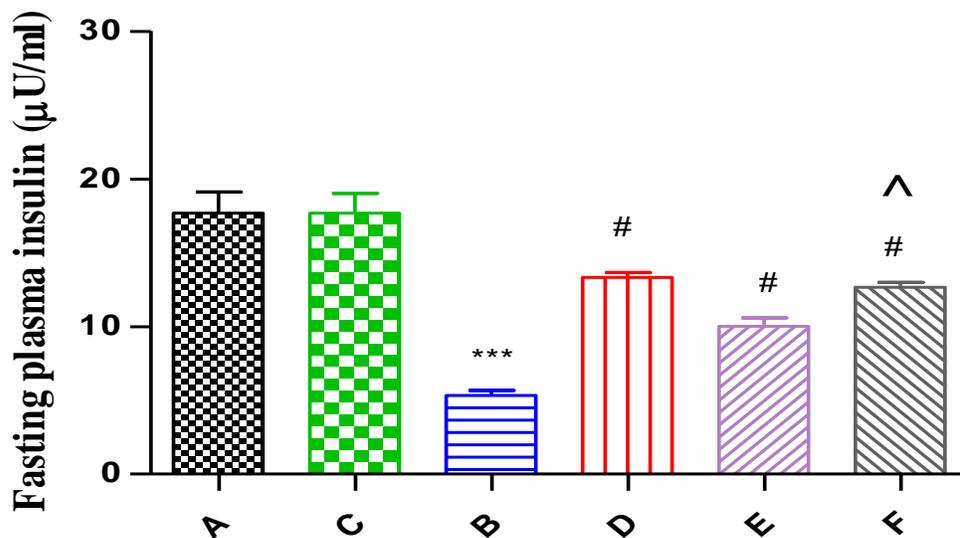
FBG concentrations were significantly ( $p < 0.05$ ) elevated in non-treated diabetic animals compared to controls. Treatment with either MO (500 mg/kg BW) or insulin (2.0 IU/kg BW, bid) significantly ( $p < 0.05$ ) reduced FBG compared to non-treated diabetic animals (Fig. 3). There were no significant differences between MO treated non-diabetic and control rats or between MO-treated (250 mg/kg BW) and non-treated diabetic rats.



**Fig. 3.** FBG concentrations measured after the animals were fasted overnight and blood drawn by tail pricking into a test strip and measured by a glucometer. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated diabetic; E= MO (250 mg/kg BW)-treated diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*, #  $p < 0.05$  compared to control (A) and non-treated diabetic group (B), respectively).

### 3.2.4 Fasting plasma insulin

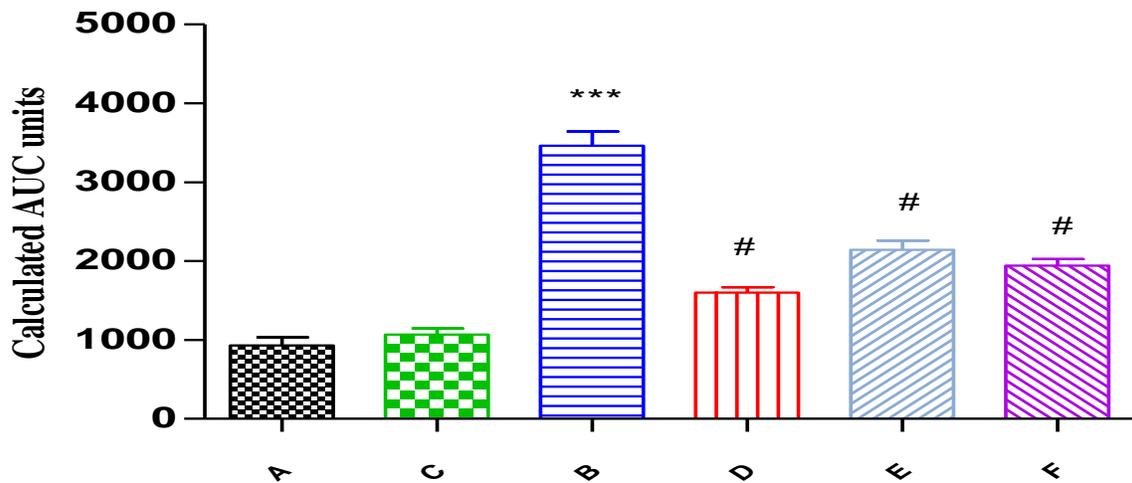
Fasting plasma insulin concentrations were significantly ( $p < 0.05$ ) reduced in non-treated diabetic rats compared to controls. Treatment with either MO or insulin (2.0 IU/kg BW, bid) significantly ( $p < 0.05$ ) improved fasting plasma insulin concentration in diabetic compared to non-treated diabetic rats. There were no significant differences between MO-treated non-diabetic and control rats (Fig. 4).



**Fig. 4.** Fasting plasma insulin measured in heparinized blood that was drawn by cardiac puncture after the animals were euthanized by halothane overdose. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated diabetic; E= MO (250 mg/kg BW)-treated diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*, #, ^  $p < 0.05$  compared to control (A), non-treated diabetic group (B) and MO (250 mg/kg BW)-treated diabetic group (E), respectively).

### 3.2.5 Glucose tolerance

Calculated AUC units from GTT suggested that untreated diabetic rats had significantly ( $p < 0.05$ ) increased glucose intolerance compared to controls. Treatment with MO or insulin (2.0 IU/kg BW) significantly reduced glucose intolerance in diabetic animals compared to the untreated diabetic group. There were no significant differences between MO treated non-diabetic and control rats (Fig. 5).

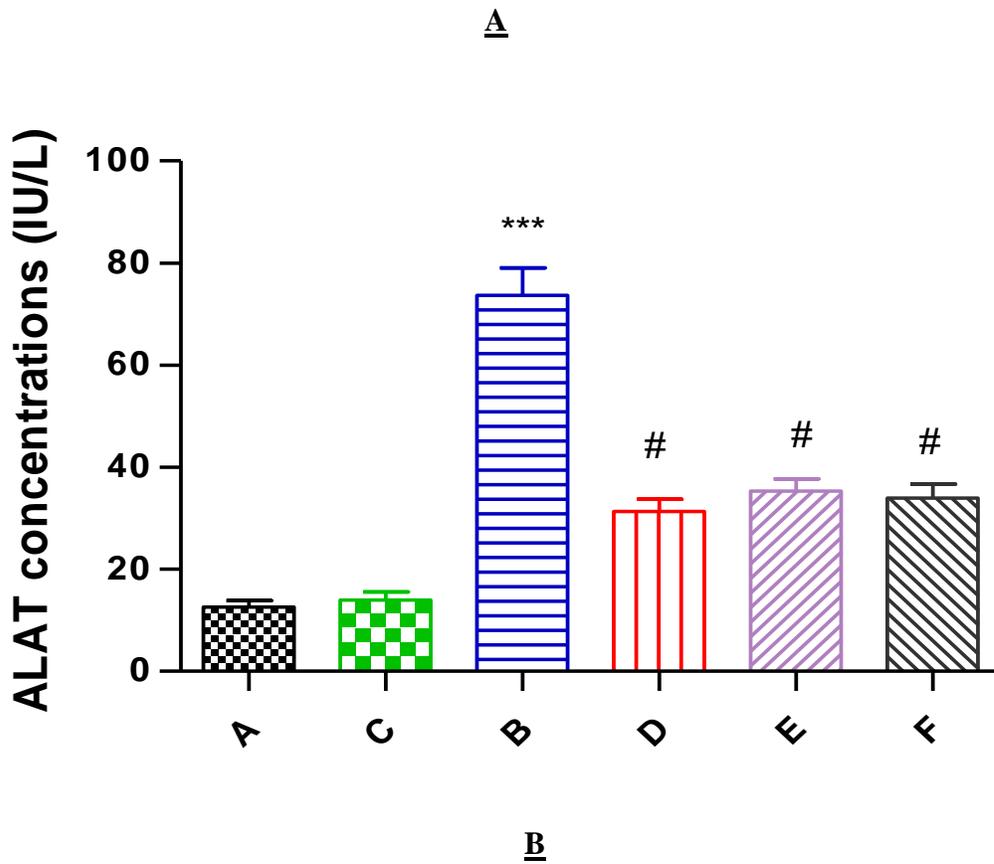


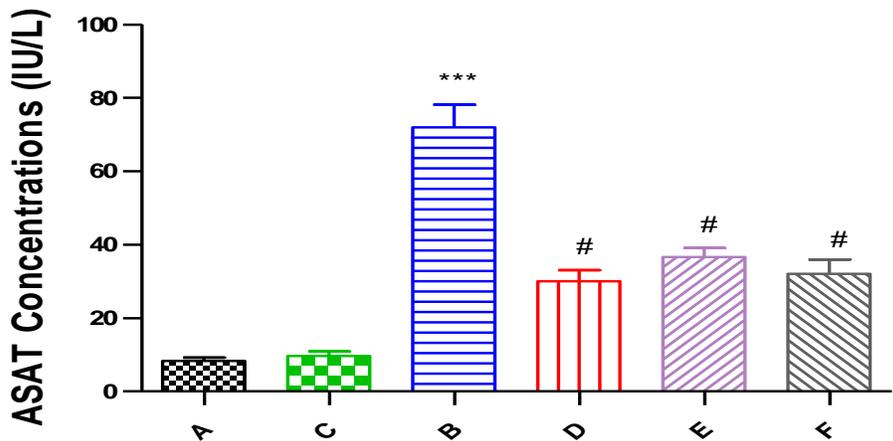
**Fig. 5.** Calculated AUCs from glucose tolerance curves in GTT. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated diabetic; E= MO (250 mg/kg BW)-treated diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*, #  $p < 0.05$  compared to control (A) and non-treated diabetic group (B), respectively).

### 3.3.0 Liver function tests

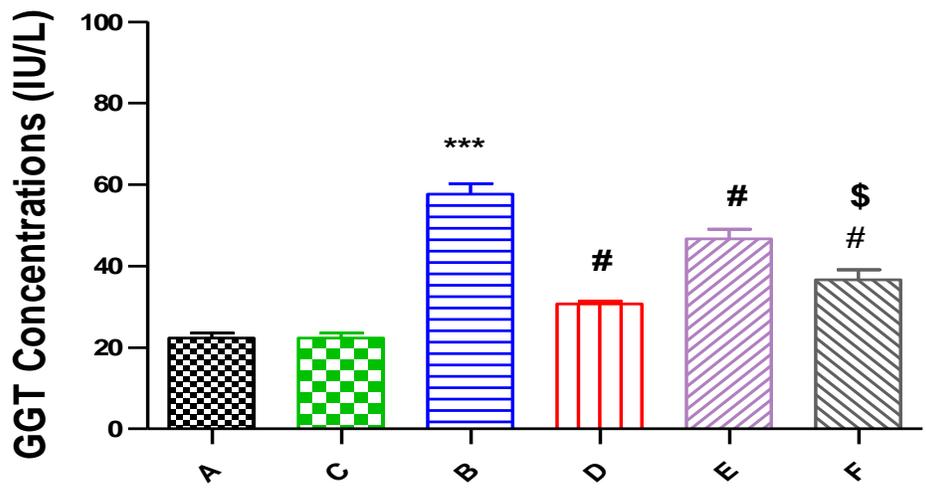
#### 3.3.1 Liver enzymes

Fasting plasma ALAT, ASAT and GGT concentrations were significantly ( $p < 0.05$ ) elevated in non-treated diabetic rats compared to controls (Fig 6A-C). Treatment with MO (either 500 or 250 mg/kg BW) or insulin (2.0 IU/kg BW) significantly reduced plasma ALAT, ASAT and GGT levels in diabetic compared to non-treated diabetic rats. Treatment with MO (500 mg/kg BW) significantly ( $p < 0.05$ ) reduced plasma GGT concentrations in diabetic rats compared to MO (250 mg/kg BW) (Fig 6C). There were no significant differences in liver enzymes between MO-treated non-diabetic and control rats.





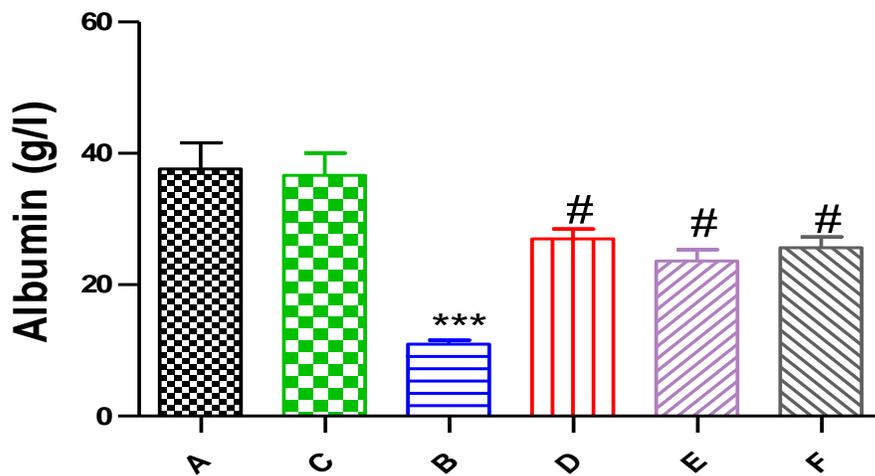
**C**



**Fig 6.** Fasting plasma **A**: ALAT; **B**: ASAT and **C**: GGT concentrations after the animals were euthanized by halothane overdose at the end of the treatment period and blood collected in heparinized tubes by cardiac puncture. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated diabetic; E= MO (250 mg/kg BW)-treated diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*, #, \$ p<0.05 compared to control (A), non-treated diabetic group (B), and MO (250 mg/kg BW)-treated diabetic group (E), respectively).

### 3.3.2 Plasma albumin

Plasma albumin concentrations were significantly ( $p < 0.05$ ) reduced in non-treated diabetic rats compared to controls. Treatment with MO (either 500 or 250 mg/kg BW) or insulin (2.0 IU/kg BW) significantly ( $p < 0.05$ ) increased plasma albumin levels in diabetic compared to non-treated diabetic rats. There were no significant differences in plasma albumin levels between MO-treated non-diabetic and control rats (Fig 7).

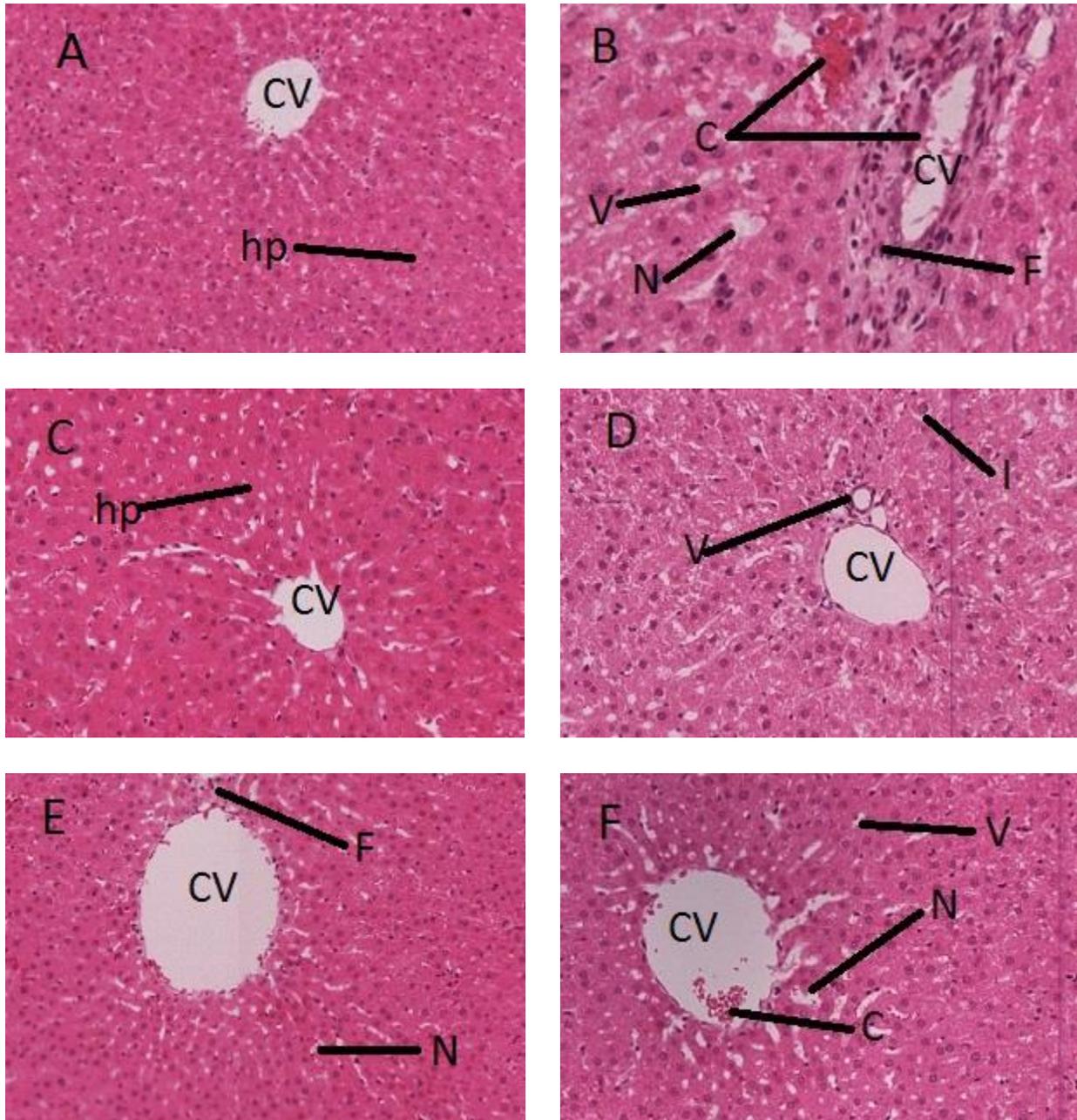


**Fig. 7.** Plasma albumin concentrations in blood that was collected by cardiac puncture in heparinized tubes at the end of the treatment period. A = controls; C= MO (500 mg/kg BW)-treated, non-diabetic; B= Non-treated, diabetic; D= Insulin (2.0 IU/kg BW, bid)-treated diabetic; E= MO (250 mg/kg BW)-treated diabetic; F= MO (500 mg/kg BW)-treated, diabetic. (\*\*\*, #  $p < 0.05$  compared to control (A) and non-treated diabetic group (B), respectively).

### 3.4.0 Histological analysis

The histopathological examination of liver sections from the control group (panel A) showed normal liver structure including central vein (CV) and hepatic cells (hp). However, non-treated diabetic rats (panel B) had livers with histopathological alterations such as degeneration of hepatocytes and inflammatory infiltrates with the fragmentation of the nucleus and cell lysis (F), necrotic hepatocytes (N), hepatic vein congestion (C), as well as vesicular cytoplasm (V). These pathological aberrations were absent in diabetic rats that were treated with either MO extracts or

insulin (panels E, F, D respectively) (Fig 8).



**Fig. 8.** Hematoxylin and eosin stains of sections of liver tissues at the end of the treatment period. Panel A = controls showing normal liver structure including central vein (CV) and hepatic cells (hp); Panel B= Non-treated, diabetic livers with degeneration of hepatocytes and inflammatory infiltrates with fragmentation of the nucleus and cell lysis (F), necrotic hepatocytes (N), hepatic vein congestion (C), as well as vesicular cytoplasm (V); Panel C= MO (500 mg/kg BW)-treated,

non-diabetic showing normal histopathology similar to controls; Panel D= Insulin (2.0 IU/kg BW, bid)-treated diabetic showing improved hepatocyte architecture; Panel E= MO (250 mg/kg BW)-treated diabetic livers showing improved hepatocyte architecture similar to the insulin-treated diabetic group and Panel F= MO (500 mg/kg BW)-treated, diabetic livers with prominent central vein (**CV**), reduced hepatic vein congestion (**C**), necrotic hepatocytes (**N**), fragmentation of nucleus and cell lysis (**F**) and vesicular cytoplasm (**V**).

#### **4.0 Discussion**

This study was designed to investigate the hepatoprotective effects of MO methanolic leaf extracts in diabetic rats. Diabetes was induced by a single intraperitoneal injection of 45 mg/kg BW of STZ which partially destroyed pancreatic  $\beta$ -cells leading to hyperglycemia (El-mahmoudy et al, 2005; Szkudelski, 2001). Our results here indicate that the animals were indeed diabetic with reduced weight gain (Fig. 1), polydipsia (Fig. 2), increased FBG (Fig. 3), reduced fasting plasma insulin (Fig. 4) and impaired glucose tolerance (Fig. 5). Treatment with MO methanolic leaf extracts (500 mg/kg BW) significantly mitigated these symptoms of diabetes (Figs. 1-5). That treatment with 250 mg/kg BW of MO methanolic leaf extracts only led to partial antidiabetic effects suggests that these pharmacological effects of MO are dose-dependent. *Moringa oleifera* contains alkaloids, flavonoids, glycoside, stilbenes and terpenoids as its bioactive compounds, which elicit their anti-diabetic effect by causing an increase in insulin sensitivity or by inhibition of the intestinal absorption glucose or to the facilitation of metabolites in insulin-dependent processes (Gupta and Misra, 2006).

This study found that insulin levels were increased in diabetic rats treated with MO extracts at different dosages (250 mg and 500 mg). This finding is in agreement with another study done by Anthanont *et al.*, (2016).

Hepatic injury associated with increased hyperglycemia has previously been documented (Mota et al., 2016; Sokolovska et al, 2015; Shima et al, 2013). In our study, degeneration of hepatocytes and inflammatory infiltrates with the fragmentation of the nucleus and cell lysis, necrotic hepatocytes, hepatic vein congestion, as well as vesicular cytoplasm were evident in non-treated diabetic rats (Fig. 8) similarly to previous reports (Kamalakkannan and Prince, 2006; Omodanisi

*et al.*, 2017). However, treatment with MO just like insulin, restored some of these histopathological aberrations, suggesting a protective role of MO in hyperglycemia-associated hepatic injury. We have presented evidence of hepatocellular injury by measuring plasma levels of key enzymes that are used clinically in liver function tests. ALAT, ASAT, and GGT were all elevated in untreated diabetic rats but treatment with either MO methanolic leaf extracts or insulin significantly reduced plasma levels of these enzymes in diabetic animals (Fig. 6) yet there were no significant differences in these enzymes' concentrations between MO-treated non-diabetic and control rats. That response in GGT levels was significantly lower with 500 than with 250 mg/kg BW of MO in diabetic animals suggests yet again that hepatoprotective effects of MO could be dose-dependent considering that, clinically GGT is a more reliable marker of hepatic injury than the other enzymes (Fig. 6C), (Wang *et al.*, 2016). ). The reduction of ALAT, ASAT, and GGT concentration after MO treatment observed in this study is in agreement with another study by Gupta *et al.*, (2014).

The increased activities of these enzymes in diabetic rats in this study may be a result of hepatocellular damage caused by oxidative stress that results in the leakage of ALAT, ASAT, and GGT from liver cells. MO may contain many bioactive chemical constituents like carotenoids, coumarins, essential oil, flavonoids, glycosides, lignans, monoterpenes, reducing sugars, resins, stibans, and saponins which could be responsible for the decrease of these enzymes (Aja *et al.*, 2014). Potentially, extract of MO could be increasing regenerative capacity of the  $\beta$ -cells to restore some of them to recover from damage caused by STZ.

Our results further show that untreated diabetic animals had significant hypoalbuminemia compared to controls suggesting hyperglycemia-associated hepatocellular injury. Treatment with either MO methanolic leaf extracts or insulin significantly restored plasma albumin levels compared to untreated diabetic animals yet there were no significant differences between MO-treated non-diabetic animals and controls (Fig. 7). Hypoalbuminemia is a strong clinical biomarker for hepatic inflammation and injury (Levitt and Levitt, 2016).

Although our study has not clearly shown the mechanisms by which MO methanolic leaf extracts could be exerting hepatoprotective effects, it has previously been suggested that phenolic compounds (apigenin, kaempferol, quercitrin, chlorogenic acid, rutin, and rhamnetin) and flavonoids abundantly present in MO extracts could exert free radical-scavenging effects that

reduce inflammation and oxidative stress (Sreelatha and Padma 2009; Ndhlala *et al.*, 2014; Ahmed and Urooj, 2009; Ramadan and Khalil, 2013). We have identified similar compounds to be present in our extracts, (Table 2) and therefore posit that MO ethanolic leaf extracts that we used in this study reduced inflammation and oxidative stress hence mitigated hyperglycemia-associated hepatic injury. Representative compounds identified by MS scans from integrated chromatogram peaks are presented in table 2. Prominent peaks are highlighted in bold. These compounds could have hepatoprotective activity. In this particular study, it is not clear if the activity resulted from a mixture of or individual compounds. Their significance as the bioactive chemical compound has not been previously determined. Hence future studies need to isolate them individually and test in vitro if they will have hepatoprotective activity.

## 5.0 Conclusions

Our study reported herein has shown that methanolic leaf extracts of MO have dose-dependent antidiabetic and hepatoprotective effects which could be attributed to its phenolic bioactive compounds. These findings, therefore, lend credence to the folkloric medicinal claims of hepatoprotective effects of MO leaf extracts by African traditional healers. Even though MO was originally from Asia, this plant has been grown in South Africa and also used in traditional medicine for the management of certain diseases, including diabetes. Further studies are needed to investigate phytochemical compounds responsible for the effects and the molecular mechanisms involved in hepatoprotective properties of MO.

## Acknowledgments

We are indebted to the staff of Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal for technical assistance. Many thanks to the University of KwaZulu-Natal, College of Health Sciences for Scholarship awarded to TMW and for running expenses for this study.

## Conflict of interest

None to declare

## Authors' contributions

WTM did the experiments and drafted the initial manuscript. MN and PO conceptualized the study and revised the manuscript for its intellectual content.

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## **CHAPTER 3: GENERAL DISCUSSION, CONCLUSION AND RECOMMANDATION.**

### **3.1 GENERAL DISCUSSION AND CONCLUSION**

This study was carried out to investigate whether *Moringa oleifera* could mitigate hepatotoxicity in diabetes mellitus.

Because of its functional roles in the regulation of physiological processes in the body, the liver is prone to toxicity. Injury to the liver may affect the integrity of hepatocytes leading to the release of liver enzymes such as ALAT, ASAT, and GGT since these enzymes are commonly used as markers of liver injuries (Harris, 2005).

In the present study, damage to the liver caused by diabetes was evident in plasma levels of ALAT, ASAT, GGT and albumin concentrations. Induction of diabetes significantly increased the plasma levels of liver enzymes (ALAT, ASAT, and GGT) and significantly decreased the plasma levels of albumin. This result indicates that acute hepatotoxicity and liver dysfunction may arise due to diabetes. After that, increase in the enzyme activities may mostly be due to leakage of these enzymes from the liver cells into the bloodstream which screen for the hepatotoxic effect of diabetes-mediated toxicity (Paremes *et al.*, 2015).

In conclusion, fasting plasma ALAT, ASAT and GGT concentrations were significantly ( $p < 0.05$ ) increased in non-treated diabetic rats compared to controls. Treatment with MO significantly decreased plasma ALAT, ASAT and GGT levels in diabetic compared to non-treated diabetic rats.

Plasma albumin concentrations were significantly ( $p < 0.05$ ) decreased in non-treated diabetic rats compared to controls. Treatment with MO significantly ( $p < 0.05$ ) increased plasma albumin levels in diabetic compared to non-treated diabetic rats.

Therefore, this result as shown by biochemical and histological analysis, support the hypothesis of this study and shows that the extract of *Moringa oleifera* grown in South Africa could mitigate hepatotoxicity associated with diabetes mellitus. This effect could be attributed to the presence of flavonoids and phenolic compounds (apigenin, kaempferol, quercitrin, chlorogenic acid, rutin, and rhamnetin) and their antioxidant effects, besides the free radical scavenging property of this plant.

### **3.2 RECOMMENDATIONS FOR FUTURE RESEARCH**

Further studies are needed to investigate phytochemical compounds responsible for the effects and the molecular mechanisms involved in hepatoprotective properties of MO.

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## APPENDIX A: Ethical approval letter.



08 September 2017

Mr Willy Tambwe Muzumbukilwa (217040227)  
School of Health Sciences  
Westville Campus

Dear Mr Muzumbukilwa,

Protocol reference number: AREC/038/017M

Project title: Hepatoprotective potential of *Moringa oleifera* leaf extracts in streptozotocin-induced diabetes in rats

### Full Approval – Research Application

With regards to your revised application received on 08 August 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and FULL APPROVAL for the protocol has been granted with the following condition:

#### CONDITION:

1. A maximum of 2 – 3 animals can be housed in each medium size polycarbonate cage.

**Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.**

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 08 September 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Prof S Islam, PhD  
Chair: Animal Research Ethics Committee

/ms

cc Supervisor: Dr Peter Owira and Dr Manimbulu Nlooto  
Cc Registrar: Mr Simon Mokoena  
Cc BRU – Dr Linda Bester

Cc Academic Leader Research: Professor Johan van Heerden  
Cc NSPCA

Animal Research Ethics Committee (AREC)

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Website: <http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx>



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## APPENDIX-B: Proof of animal training.



### BIOMEDICAL RESOURCE UNIT

May 3, 2017

Dear Prof Islam  
Chair: Animal Research Ethics Committee  
c/o School of Life Sciences

#### RE: ATTENDANCE OF LAS COURSE

This letter certifies that Mr Willy Tambwe Muzumbukilwa have attended the Laboratory Animal Course that was hosted by the Biomedical Resource Unit.

The course was held on the 16 – 17 of March 2017 and entailed the following:

Introduction to laboratory animal sciences. Bioethics and Animal experimentation.  
Animal Research Methodology. Experimental design, environmental enrichment and occupational safety.

The course was completed satisfactorily and may be allowed to initiate his research after the relevant practical procedures was done to a level of competency that was signed off by the veterinarian in charge.

Kind Regards

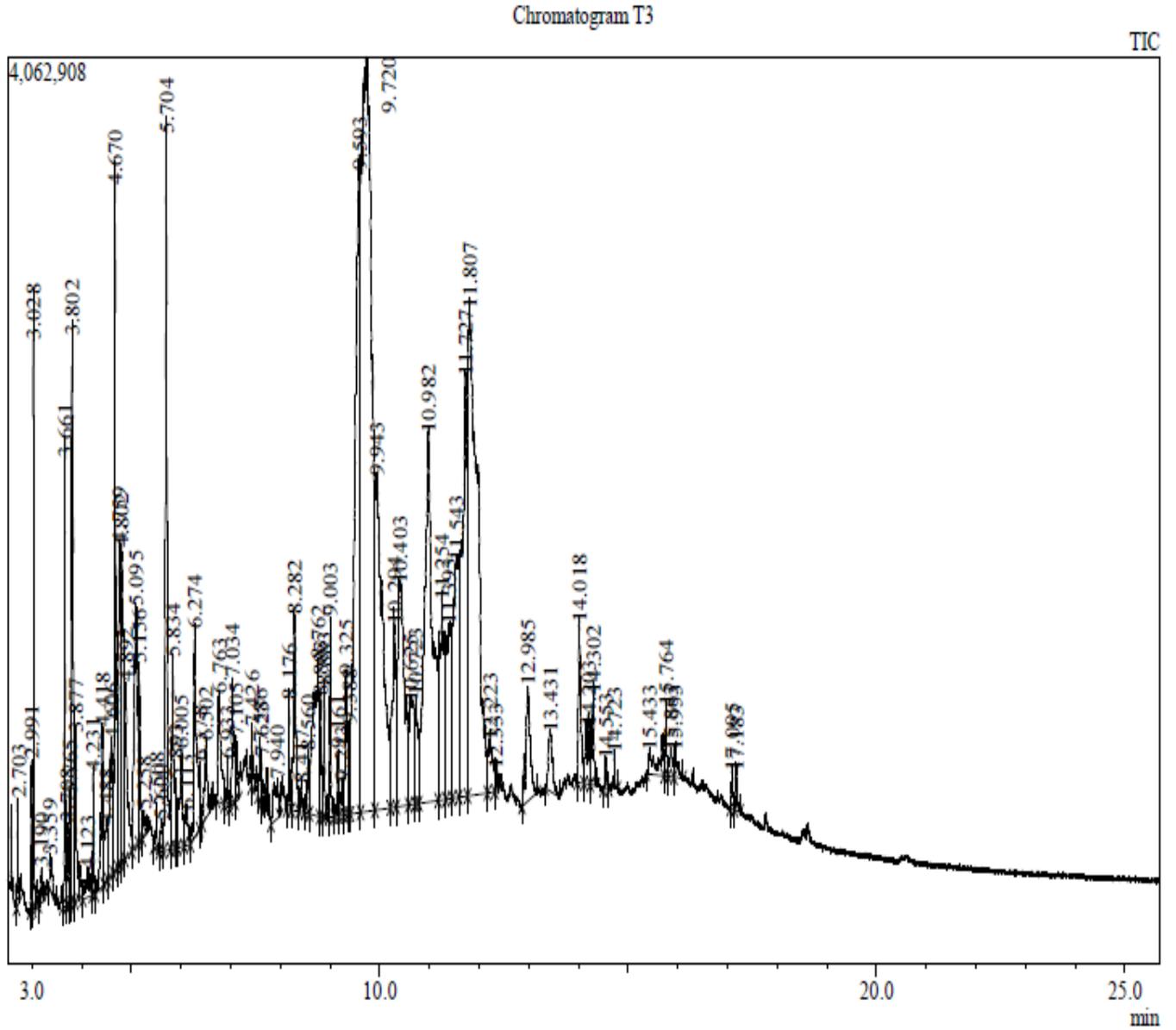
A handwritten signature in black ink, appearing to read "Dr Singh".

Dr SD Singh BVSc. (Mumbai) MS (Illinois) LAS (Utrecht) CVE (Pretoria)  
HOD: Biomedical Resource Unit  
Veterinarian

Miss Ritta Radebe, Room 201, 2nd Floor U- Block ,Tel 031 260 7671,Fax 031 260 7730 E-mail [radeber@ukzn.ac.za](mailto:radeber@ukzn.ac.za)

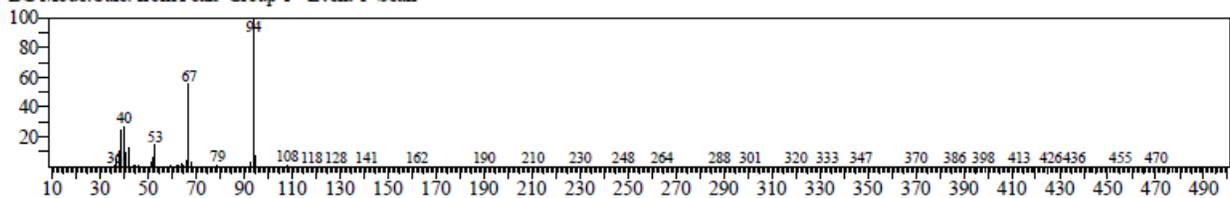
**REDUCE, REFINE AND REPLACE**

APPENDIX-C: GC-MS chromatogram for *Moringa oleifera*.



<< target >>

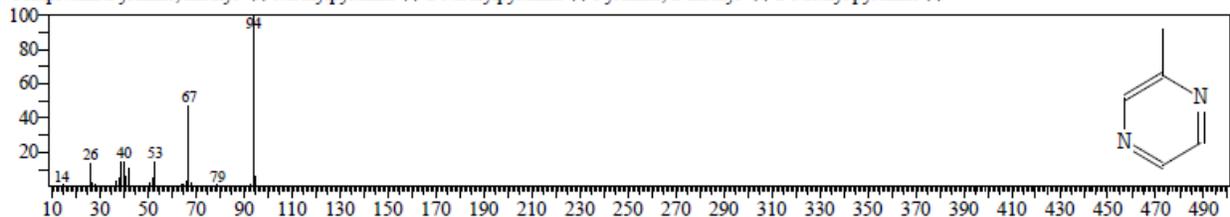
Line# 2 R Time: 2.990 (Scan#: 295) MassPeaks: 213  
RawMode: Averaged 2.988-2.992 (294-296) BasePeak: 94.05 (211780)  
BG Mode: Calc. from Peak Group 1 - Event 1 Scan



Hit# 1 Entry: 1261 Library: NIST11.lib

SI: 94 Formula: C<sub>5</sub>H<sub>6</sub>N<sub>2</sub> CAS: 109-08-0 MolWeight: 94 RetIndex: 781

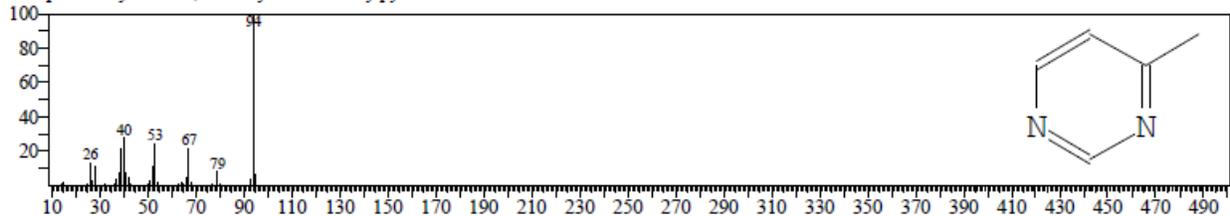
CompName: Pyrazine, methyl- \$\$ Methylpyrazine \$\$ 2-Methylpyrazine \$\$ Pyrazine, 2-methyl- \$\$ 2-Methyl pyrazine \$\$



Hit# 2 Entry: 1255 Library: NIST11.lib

SI: 92 Formula: C<sub>5</sub>H<sub>6</sub>N<sub>2</sub> CAS: 3438-46-8 MolWeight: 94 RetIndex: 781

CompName: Pyrimidine, 4-methyl- \$\$ 4-Methylpyrimidine \$\$



Representative's chromatogram.

## APPENDIX-D: SUPPLEMENTARY PAPER

**Title:** Mapping the evidence of hepatoprotective properties of *Moringa oleifera* from sub-Saharan African countries: a systematic review protocol.

**Authors:** Willy Tambwe Muzumbukilwa<sup>1\*</sup>, Mukanda Gedeon Kadima<sup>1</sup>, Manimbulu Nlooto<sup>1</sup>, Peter Mark Oroma Owira<sup>1</sup>.

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## Abstract

**Background:** One of the most challenging health problems is liver disease, which can be caused by medications, toxic substances, and excessive consumption of alcohol. Liver problems can be also caused by infections, autoimmune disorders, and food. This study aims to establish evidence of the use of *Moringa oleifera* sub-Saharan African countries to manage liver damage, conditions in animals.

**Methods:** *In vivo* studies will include those in which the activity of the serum levels of hepatic enzymes Alanine Transaminase (ALT) or serum glutamate-pyruvate transaminase (SGPT), Aspartate Transaminase (AST) or Serum Glutamic oxaloacetic transaminase (SGOT), Gamma-Glutamyltransferase (GGT) and/or Alkaline phosphatase (ALP) were measured after administering substances that induced liver injury as a primary outcome.

The secondary outcome will include studies that measure the serum levels of hepatic enzymes (ALT, AST, GGT, and ALP) after administering the sub-Saharan *Moringa oleifera*, decreases in their levels indicating the improvement of their activity.

Articles written in languages other than English and French will be excluded. Search engines will include MEDLINE, CINAHL, PubMed and Google Scholar. The screened results will be grouped according to any noteworthy grouping variable, such as study characteristics.

**Discussion:** The results will give scientists new insight into the possible use of this plant to develop effective hepatoprotective drugs treat liver damage, and indicate areas of research for consideration.

**Systematic review registration:** CRD42018084698.

**Keywords:** *Moringa oleifera*, alanine aminotransferase, aspartate aminotransferase, Gamma-glutamyltransferase, alkaline phosphatase, sub-Saharan Africa countries.

## Background

The liver plays a major role in regulating many of the body's physiological processes such as its metabolic, vascular, immunological, storing, secretory and excretory functions. Furthermore, it is involved in detoxifying a variety of drugs and xenobiotics; and plays a key role in metabolizing

carbohydrate, protein, and fat in the human body [1]. However, the ability of the liver to achieve these functions is often inhibited by many substances which may cause liver diseases. Liver diseases are some of the most challenging health problems, which can be caused by the undesirable effects of some medications, toxic substances, excessive consumption of alcohol, infections and autoimmune disorders [2]. Liver diseases, which can cause death include cirrhosis, liver cancer, viral hepatitis (B, C, and D), non-alcoholic fatty liver diseases (NALD), alcoholic liver disease, autoimmune and drug-induced liver diseases [3]. The prevalence of non-alcoholic fatty liver disease was estimated to 20% worldwide in the general population in the year 2012 [4]. Two billion people were infected by hepatitis B worldwide in 2015, and 520,000 deaths were reported, with 170 million being affected by hepatitis C [5]. In India, almost 20,000 deaths are reportedly due to liver diseases every year [6]. Europe reported 63,500 cases of liver cancer in the year 2012, with 29 million people suffering from liver diseases in 2013. In the United States of America, 30 million people suffered from liver diseases versus 2013, with 170,000 deaths being reported due to liver cirrhosis [5].

Alanine Transaminase (ALT), Aspartate Transaminase (AST) and Gama-Glutamyltransferase (GGT) are serum of hepatocellular enzymes that are normally found in the liver. ALT is the specific marker for liver injury, AST can provide information about other tissues including the heart and skeletal, while GGT is found in the kidney and pancreas [7]. The elevated levels of these enzymes in the serum is a sign of their increased entrance in serum from damaged liver cells<sup>6</sup>, with injury being identified using enzymes as hepatic markers [8]. Studies have shown that the chances of the damaged liver being able to heal are reduced in the acute phase which often leads to chronic diseases with complications [9]. In spite of considerable progress in modern medicine, there are very few therapeutic agents that can protect the liver from damage and stimulate liver functions [10]. Patients with chronic liver disease are liable to have liver transplantation, which is not only costly but has long-term consequences of immunosuppressive agents, especially hyperlipidemia, hypertension and renal disease [11]. For these reasons, many patients with liver disease use herbal remedies to solve their health problems [12].

*Moringa oleifera* is cultivated worldwide, but native of India, Bangladesh, and Pakistan [13], being a wild and domestic tree in many sub-Sahara African countries. Phytochemical analyses have shown that its leaves contain various minerals, such as calcium, iron, phosphorous, potassium and vitamins (A, C and D) [14]. Its bark, flowers, fruits, leaves, pods, and roots possess many

biological and pharmacological properties, such as antibacterial, antidiabetic, anti-inflammatory, antifungal, antihypertensive, antioxidant, antipyretic, antispasmodic, antitumor, antiulcer, cholesterol lowering, diuretic and hepatoprotective activities [9]. The leaf extracts reportedly reduce serum intracellular enzymes levels by stabilizing the cell membrane, which prevents enzymes leakages [15]. While the agro-climatic conditions of vegetable materials may vary from one place to another; little is known about the different species of *Moringa* in sub-Saharan countries. This study, therefore, aims to establish the evidence of *Moringa oleifera* from sub-Saharan Africa being used to manage liver damage, specifically in animals. The results will give scientists new insights into the possible use of this plant to develop effective hepatoprotective drugs against liver damage.

### **Research questions**

For the purpose of this study, the general research question is as follows: Does *Moringa oleifera* from sub-Saharan African manage liver damage? To answer this general question, specific research questions have been developed:

1. Does the administration of substances inducing liver damage increase the levels of hepatic enzyme activities (ALT, AST, GGT and/or ALP);
- 2 Does *Moringa oleifera* from sub-Saharan African decrease the activity of hepatic enzymatic markers?

### **The study objectives are:**

- 1 To establish whether substances inducing liver damage increase the levels of hepatic enzyme activities (ALT, AST, GGT and/or ALP).
- 2 To determine whether *Moringa oleifera* decrease the activity of the hepatic enzymatic markers.

### **Methods/ design**

This systematic review is reported following the Preferred Reporting Items for Systematic Reviews and Meta-analyses Protocols (PRISMA-P) 2015 checklist (Additional File 1) [16]. This review protocol having been registered in the international prospective register of the systematic review (PROSPERO), register number CRD42018084698.

**Population**

Studies using rats and mice will be selected. Also, studies using *Moringa oleifera* growing in sub-Saharan African countries will be selected.

**Intervention**

The intervention will include studies in which administration of substance was done to induce liver damage in rats and mice. The intervention group will include also studies in which *Moringa oleifera* from sub-Saharan African was administered to treat the induced liver damage.

**Comparison**

The comparison will include studies in which groups of animals that have and have not been treated with *Moringa oleifera* from within African were compared. This comparison will be evaluated according to the level of liver markers such as ALT, AST, GGT and/or ALP.

**Types of outcomes****Primary outcome**

The primary outcome will consist of studies in which the levels of hepatic enzymes activities of ALT, AST, GGT and ALP in the serum after administration of substances that induced liver damage were measured. Increase in the levels of those hepatic enzymes in serum would indicate damaged liver possibly caused by these substances.

**Secondary outcome**

The secondary outcome will consist of studies in which the levels of hepatic enzymes activities of ALT, AST, GGT and/or ALP in serum after administration of sub-Saharan African *Moringa oleifera* were measured. This can have positive, negative, or no effects when compared to standard treatment before and after the intervention. Decreases in the levels of those hepatic enzymes in the serum would indicate an improvement of their activity after the administration of *Moringa oleifera* (positive effect). Increases in the levels of those hepatic enzymes in the serum would indicate no improvement in their activity after the administration of *Moringa oleifera* (negative effect). Lastly, when there is no change of hepatic enzymes levels when compared to standard treatment before, the case effects will be regarded as static.

## **Study eligibility criteria**

### **Inclusion Criteria**

Studies will be included in the systematic review if they satisfied all of the following:

- English and French research published before December 31, 2018.
- *In vivo* research on rats and mice treated with sub-Saharan African *Moringa oleifera* to manage the liver damage.
- Where AST, ALT, GGT, and ALP are measured in the animals' serum.
- Case-control groups of treatment with and without *Moringa oleifera*.

### **Exclusion criteria**

- Articles on human or animal study other than rats and mice.
- Letters, reviews, case report and comments.
- All articles without available full text.
- Articles not addressing the hepatoprotective activity of *Moringa oleifera*.
- Articles using co-administration with another plant.
- Articles using *Moringa oleifera* not harvested in sub-Saharan Africa.
- Case-control group of treatment with another plant than *Moringa oleifera*.

### **Information sources and search strategy for identifying relevant studies**

A comprehensive and exhaustive search of CINAHL, Google Scholar, Medline, and PubMed Database will be performed to identify all relevant articles published on hepatoprotective properties of *Moringa oleifera* from sub-Saharan African countries before December 31, 2017, regardless of the language of publication. A search strategy based on the combination of relevant terms will be conceived and applied. The following terms and their variants will be used for *Moringa oleifera*: “*Moringa oleifera*”, “Morungue”, “Drumstick tree”, “Horseradish tree”, “Ben tree”, “Moringueiro” and “Rawag”. For hepatoprotective, we will use the terms “Alanine Transaminase”, “Alamine Amino Transferase”, “serum glutamate-pyruvate transaminase”, “Aspartate Transaminase”, “Aspartate Amino Transferase”, “Serum Glutamic oxaloacetic transaminase”, “Gama-Glutamyltransferase” and “Alkaline phosphatase”. Individual country names for the 54 sub-Saharan African countries will also be used as additional key search terms for more abstracts on the subject. African country names will be introduced both in English and

languages relevant to each country for example, “Ivory Coast” and “Côte d’Ivoire”. Where country names have changed over time, old and new names will be included, such as “Zaire” and “Democratic Republic of Congo”. Abstracts of all eligible papers will be reviewed and full texts of articles will be accessed through CINAHL, Google Scholar, MEDLINE and PubMed. The main search strategy conducted in PubMed is shown in Table 1. This search strategy will be adapted for searching other databases.

A manual search which consists of scanning the reference lists of eligible papers and other relevant review articles will be also conducted.

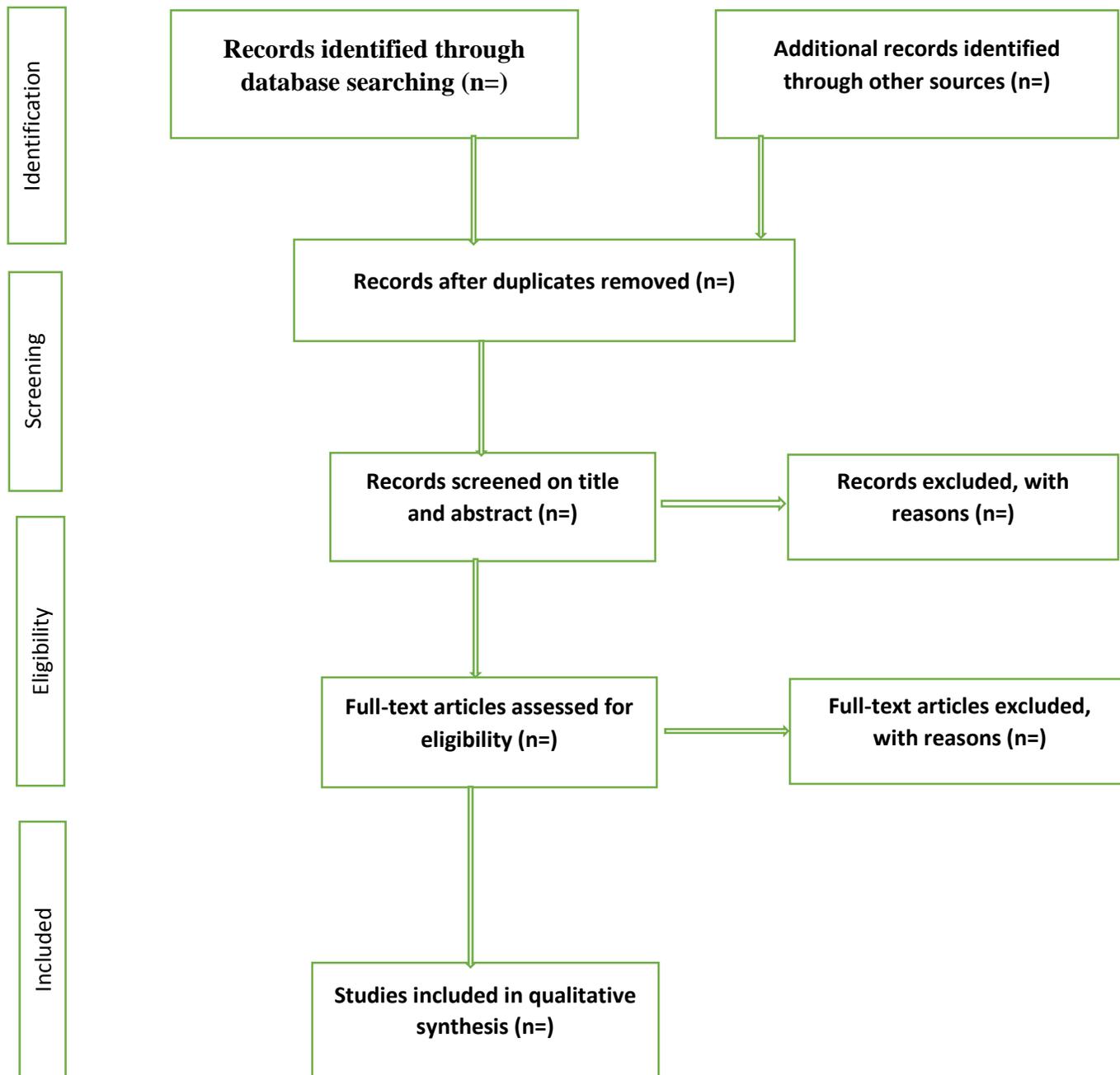
**Table 1: Search strategy in PubMed**

<b>Search</b>	<b>Area</b>	<b>Search terms</b>
<b>#1</b>	Moringa	Moringa oleifera OR Morungue OR Drumstick tree OR Horseradish tree OR Ben tree OR Moringueiro OR Rawag
<b>#2</b>	Hepatoprotective	Alanine Transaminase OR Alamine Amino Transferase OR serum glutamate-pyruvate transaminase OR Aspartate Transaminase OR Aspartate Amino Transferase OR Serum Glutamic oxaloacetic transaminase OR Gama-Glutamyltransferase OR Alkaline phosphatase.
<b>#3</b>	Sub-Saharan Africa	(Algeria OR Angola OR Benin OR Botswana OR Burkina Faso OR Burundi OR Cameroon OR Canary Islands OR Cape Verde OR Central African Republic OR Chad OR Comoros OR Congo OR Democratic Republic of Congo OR Djibouti OR Egypt OR Equatorial Guinea OR Eritrea OR Ethiopia OR Gabon OR Gambia OR Ghana OR Guinea OR Guinea Bissau OR Ivory Coast OR Cote d'Ivoire OR Jamahiriya OR Kenya OR Lesotho OR Liberia OR Libya OR Madagascar OR Malawi OR Mali OR Mauritania OR Mauritius OR Mayotte OR Morocco OR Mozambique OR Namibia OR Niger OR Nigeria OR Rwanda OR Sao Tome OR Senegal OR Seychelles OR Sierra Leone OR Somalia OR South Africa OR St Helena OR Sudan OR Swaziland OR Tanzania OR Togo OR Tunisia OR Uganda OR Western Sahara OR Zaire OR Zambia OR Zimbabwe OR sub Saharan Africa).
<b>#4</b>		#1 AND #2 AND #3

### **Selection of studies**

All studies identified using selected keywords will be screened after removing duplicated articles. The first choice of inclusion criteria for this study will be based on titles and thereafter on abstracts if the title does not clearly give the information related to our study. Full articles will be found for detailed appreciation against the inclusion criteria before screened of articles. Finally, included articles for the study will be selected in using the above eligibility criteria. Assessment of studies for possible inclusion will be done by two reviewers, and any changes of views will be fixed through discussion until a compromise is reached. Any papers that are not unanimously excluded or included by both reviewers will be examined by both reviewers until an outcome is agreed. If necessary, a third person may be consulted. This will reduce the risk of bias. The review study selection process will be followed by a flow diagram showing the number of all excluded studies as well as reasons for their exclusion (Figure 1) [17].

Figure 1: PRISMA-P flow-chart of the study selection procedure.



**Data extraction and management.**

A data extraction form will be created by the principal author (WTM) in Microsoft Excel and modified by feedback from three independent reviewers (MGK, MN, and PMOO) to ensure that complete data is obtained. Two review authors (WTM and MGK) will independently extract data. Data will be collected on the first author name; animal species (rats, mice), gender (male and/or female), age (mature adult, middle-aged, old adult), weight; nature of acute liver damage (drug, toxic chemical, alcohol, food, and infection or autoimmune induced damage); part of Moringa used (bark, flour, leaves, seed), African country of harvest, dose of Moringa administered, duration of treatment; and primary and secondary outcome measurements.

Disagreements between authors will be resolved through discussion and consensus, or arbitration by a third author whenever necessary. For managing missing data, we will contact the corresponding author of the respective studies in order to obtain the required details. If no correspondence is received, the study will be included in the systematic review and discussed in the narrative summary.

**Data analysis including an assessment of heterogeneity**

Data will be analyzed using Stata statistical software (Stata Corp V.14, Texas, USA).

Study data will be quantitatively synthesized by first assessing heterogeneity to examine whether the estimates from included studies could be pooled. Heterogeneity will be assessed by the Chi-squared test on Cochran's Q statistic, which will be quantified by I-squared values. Values of 25, 50, and 75% for I-squared will be represented low, medium, and high heterogeneity, respectively [18]. When substantial heterogeneity will be detected, we will perform meta-regression and subgroup analyses to investigate the possible sources of heterogeneity using the following grouping variables: animal species (rat , mice), age (mature adult, middle-aged, old adult), gender (male, female, combination of genders used), weight; nature of acute liver damage (drug, toxic chemical, alcohol, food, and infection or autoimmune induced damage); part of Moringa used (Bark, flour, leaves, seed), African country of harvested, dose of Moringa administered, duration of treatment; and outcome measurement results. The heterogeneity between subgroups will be detected by using the  $X^2$  test on Cochran's Q statistic. In cases where quantitative synthesis is not appropriate because it is not possible to conduct meta-analysis due to the heterogeneity of estimates reported by studies, a qualitative narrative synthesis of the evidence will be performed.

This will summarize the studies characteristics, animal's features, nature of liver damage, Moringa features an outcome result.

### **Assessment of risk of bias**

The study quality and the presence of potential bias within individual studies will be done at both the outcome level and the study level. Two reviewers (WTM and MGK) will complete the assessment independently. The Cochrane risk of bias tool will be used to assess publication bias, addressing assessments for random sequence generation, concealment of allocation, blinding and the outcome measurements, and completeness of outcome reporting [19]. Each bias criterion will be assigned a value of low, high, or unclear risk of bias for each included study.

Visual assessment of the funnel plot and the Egger's statistic will be used to assess for both the presence and statistical significance of publication bias across studies [20].

### **Assessment of methodological and evidence qualities.**

We will use the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology to assess the quality of evidence for each outcome as recommended in the Cochrane Handbook for Systematic Reviews of Interventions [21]. Quality rating of overall evidence will be downgraded according to the following factors: Risk of bias, the inconsistency of result, indirectness, imprecision, and publication bias. In addition and where appropriate, the reasons to upgrade the evidence quality will include a large magnitude of effects, a dose-response gradient, and plausible residual confounding that would reduce a demonstrated effect or suggest a spurious effect when results show no effect. We will integrate downgrading and upgrading factors to obtain an overall quality of evidence for each outcome of interest. Overall quality of evidence will be then ranked as high, moderate, low, or very low as specified by the GRADE approach [21; 22].

### **Discussion**

*Moringa oleifera* (MO) is a plant traditionally used for its medicinal and nutritional properties in Africa. Many studies have been done on MO, and some of them showed that MO possesses hepatoprotective activity [1]. But then, not many studies have focused on MO from sub-Saharan countries in managing acute liver damage induced. This protocol states the plan for a systematic review and meta-synthesis of the effectiveness of *Moringa oleifera* from sub-Saharan African

countries to manage liver damage. Therefore, this review will fill this gap by revealing evidence that *Moringa oleifera* from different countries of sub-Saharan Africa have the same effects on induced acute liver damage, so agro-climatic conditions of vegetable materials may vary from one place to another [23]. Results will be shared with the scientific community through publishing in a peer-reviewed journal. The findings could give scientists new insight into the possible use of this plant to develop effective hepatoprotective drugs that treat liver damage and can serve as valuable inputs for future research in this area.

### **Abbreviations**

**ALP:** alkaline phosphatase, **ALT:** alanine Transaminase, **AST:** aspartate Transaminase, **GGT:** Gamma-glutamyltransferase, **NALD:** Non-alcoholic liver diseases, **PRISMA:** Preferred Reporting Items for Systematic Review and Meta-analyses, **PRISMA-P:** Preferred Reporting Items for Systematic Review and Meta-Analysis Protocol, **SGOT:** Serum Glutamic oxaloacetic transaminase, **SGPT:** serum glutamate-pyruvate transaminase (SGPT), **WHO:** world health organisation.

### **Declarations**

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#### **Funding**

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#### **Availability of data and material**

All data generated or analyzed during this study will be included in the published systematic review.

### **Authors' contributions**

WTM conceived and designed the protocol and drafted and revised the manuscript for intellectual content. MGK designed the protocol and revised the manuscript for intellectual content. MN supervised and revised the manuscript for intellectual content. PMOO supervised and revised the manuscript for intellectual content. All authors had full access to the data and they gave their approval before the submission of the final version to the journal.

### **Competing interests**

No, any competing interests are declared by the authors.

### **Consent for publication**

Not applicable

### **Ethics approval and consent to participate**

Not applicable

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