EFFECTS OF HYDROETHANOLIC *MORINGA OLEIFERA* LAM. SEED EXTRACT ON HIGH-FRUCTOSE DIET FED GROWING SPRAGUE-DAWLEY RATS

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, School of Physiology, in fulfilment of the requirements for the degree of Master of Science in Medicine.

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DECLARATION

I, **Masiline Mapfumo** declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University and where I have used supporting research findings these have been acknowledged accordingly. All procedures used in the process of data collection were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number 2016/10/44C).

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Masiline Mapfumo

Signed on the …….…… day of ……..… 2020

DEDICATION

To Shalom, Shanessa and Adiel Mapfumo.

RESEARCH OUTPUTS

Published Articles

- **Mapfumo M.,** Lembede, B.W., Nkomozepi, P., Ndhlala, AR., & Chivandi, E (2019). Crude *Moringa oleifera* Lam. seed extract attenuates non-alcoholic fatty liver disease in growing Sprague-Dawley rats. *South African Journal Botany*. ***Available online.***

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Conference Presentations

- **Mapfumo M.,** Lembede B.W., Ndhlala A.R., Chivandi E. *Moringa oleifera* seed extract protects growing male Sprague Dawley rats against high-fructose diet-induced hypercholesterolemia. Oral presentation at the University of the Witwatersrand, Faculty of Health Sciences Research Day, 6th September 2018, Johannesburg, South Africa.

ABSTRACT

Fructose consumption contributes to the development of non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MetS). The anti-obesogenic and hepatoprotective properties of crude *Moringa oleifera* seed extract make it a potential prophylactic ethnomedicine against diet-induced NAFLD and MetS. This study interrogated the potential of crude hydroethanolic *M. oleifera* seed extract to protect against high-fructose diet-induced metabolic dysfunction.

Eighty-eight 21-day old female and male Sprague-Dawley rat pups were randomly allocated to and administered one of the following five treatment regimens (8 female rats and 9-10 male rats per treatment) daily for twelve weeks: group I - plain drinking water (PW) + plain gelatine cube (PC), group II - 20% (w/v) fructose solution (FS) + PC, group III - FS + 100 mg/kg body mass fenofibrate in gelatine cube (FN), group IV - FS + low dose (50 mg/kg body mass) of *M. oleifera* in gelatine cube (LMol) and group V - FS + high dose (500 mg/kg body mass) of *M. oleifera* in gelatine cube (HMol). The rats in each treatment regimen had *ad libitum* access to a standard rat chow and were weighed twice weekly. At the end of the 12-week trial, the rats were subjected to an oral glucose tolerance test and then euthanised 48 hours later. Blood was collected via cardiac puncture and plasma was harvested. Plasma triglyceride, cholesterol and insulin concentration, HOMA-IR, and surrogate biomarkers of liver function were determined. The livers and visceral fat pads were dissected out, weighed and preserved for liver lipid content and histology analysis.

The consumption of a high-fructose diet increased ($p<0.05$) liver lipid content and caused steatosis and hypertriglyceridemia in female and male rats. Crude hydroethanolic *M. oleifera* seed extract and fenofibrate prevented the high-fructose diet-induced liver lipid accretion and steatosis in female and male rats. High-fructose diet increased ($p<0.05$) visceral fat pad mass, plasma insulin concentration and HOMA-IR in female rats only. The crude hydroethanolic *M. oleifera* seed extract prevented the high-fructose diet-induced hyperinsulinemia and insulin resistance in female rats but failed to prevent the visceral adiposity. Crude hydroethanolic *M. oleifera* seed extract did not compromise liver function.

The crude hydroethanolic *M. oleifera* seed extract can potentially be exploited as prophylaxis against diet-induced fatty liver disease and MetS without causing liver toxicity in children.

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(Philippians 4:13 "I can do all things through Christ who strengthens me").

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α:	Alpha
β:	Beta
AESC:	Animal Ethics Screening Committee
AGEs:	Advanced glycation end products
ALP:	Alkaline phosphatase
ALT:	Alanine transaminase
ARC:	Agricultural Research Council
AUC:	Area under the curve
AST:	Aspartate transaminase
ANOVA:	Analysis of variance
BMI:	Body mass index
CAS:	Central animal service
CIA:	Central Intelligence Agency
ELISA:	Enzyme-linked immunosorbent assay
FFA:	Free fatty acids
FN:	Fenofibrate gelatine cube
FS:	20% fructose solution
GBD:	Global Burden of Disease
GIT:	Gastrointestinal tract
GLUT:	Glucose transporter
H&E:	Haematoxylin and Eosin
HDL:	High-density lipoprotein
HFCS:	High fructose corn syrup
HMol:	High dose of *Moringa oleifera* seed extract
HOMA-IR:	Homeostatic model of insulin resistance
IDF:	International Diabetes Federation
IL-6:	Interleukin-6
iNOS:	inducible nitric oxide synthase
LCAT:	Lecithin cholesterol acyltransferase
LDL:	Low-density lipoprotein
LMol:	Low dose of *Moringa oleifera* seed extract

MetS:	Metabolic syndrome
NAFLD:	Non-alcoholic fatty liver disease
NAS:	Non-alcoholic fatty liver disease activity score
NASH:	Non-alcoholic steatohepatitis
NEFA:	Non-esterified fatty acids
NO:	Nitric oxide
OGTT:	Oral glucose tolerance test
OLETF:	Otsuka Long Evans Tokushima Fatty
PC:	plain gelatine cubes
PW:	plain water
PPAR:	Peroxisome proliferator-activated receptor
ROS:	Reactive oxygen species
SA:	South Africa
SADHS:	South African Demographic and Health Survey
SD:	Standard deviation
SRC:	Standard rat chow
SSA:	Sub-Saharan Africa
TNF:	Tumour necrosis factor
TG:	Triglycerides
TGF:	Transforming growth factor
T-II-DM:	Type-II-diabetes mellitus
VLDL:	Very low-density lipoprotein
VOPI:	Vegetable and Ornamental Plant Institute
WC:	Waist circumference
WHR:	Waist-hip ratio
WHO:	World Health Organisation
w/v:	weight/volume
w/w	weight/weight

CHAPTER 1: INTRODUCTION

1.0 Introduction

Obesity, one of the major health issues confronting humanity globally, refers to the excessive accumulation of fat that can result in adverse health outcomes (Ahmed et al., 2014). According to the report on Global Burden of Disease (GBD, 2017), as of 2015 the worldwide prevalence of childhood obesity stood at 5% and in adults at 12%, translating to 108 million and 604 million children and adults respectively. However, as reported by the World Health Organisation (WHO), over 650 million adults, 340 million children and adolescences (5-19 years) are obese (WHO, 2018). In sub-Saharan Africa (SSA), South Africa (SA) is reported to have the highest prevalence of childhood obesity at 7.8% (Toriola et al., 2012).

Numerous studies have revealed several risk factors for obesity, among them the mismatch between energy intake and expenditure (Mukhopadhyay, 2012; Roh and Jung, 2012), dietary (the consumption of high-fructose and high-fat foods), environmental (cultural, socioeconomic) and genetic factors as well as sedentary lifestyles (Bray and Popkin, 2013; Mohamed et al., 2014; Seyssel et al., 2016). Dietary fructose is consumed in condiments, desserts and carbonated beverages (Bray and Popkin, 2013; Le, 2010) while the convenience of fat-laden "take away foods" has and continues to increase the consumption of abnormally large amounts of dietary fat (Raatz et al., 2017). These poor dietary choices and inadequate physical activity have been shown to result in increased accretion of adipose tissue that results in obesity (Nettleton et al., 2014; Raatz et al., 2017). Obesity is a key risk factor to the development of metabolic derangements inclusive of hyperglycaemia, insulin resistance, dyslipidaemias and a host of non-communicable diseases including metabolic syndrome (MetS), type-II diabetes mellitus (T-II-DM), cardiovascular disease, hypertension and non-alcoholic fatty liver disease (NAFLD) in both adults and children (Hu and Malik, 2010; Matson and Fallon, 2012). Metabolic syndrome is a complex multifactorial condition which includes several metabolic disorders inclusive of abdominal obesity, atherogenic dyslipidaemia, hyperinsulinemia, hyperglycaemia, hypertension and impaired glucose tolerance (Rossouw et al., 2012; Tune et al., 2017).

Several conventional pharmacological agents are available for the management of obesity and metabolic disorders related to MetS (Mahajan and Mehta, 2010). Pharmacological agents available are inclusive of metformin used for management of

diabetes-related complications (Matson and Fallon, 2012) and fenofibrate (Kraja et al., 2010) used for the management of obesity-related complications. These conventional pharmacological agents elicit side effects (Bray, 2013). Notably, apart from causing adverse side effects, the conventional medicines are costly, inaccessible to some low- and middle-income communities (Cameron et al., 2011) and target specific disorder and not a wide range of derangements in metabolic syndrome. Consequently, due to their being natural (hence deemed safe), availability, accessibility and a multiplicity of beneficial biological activities, ethnomedicines have now become the focus in the management of metabolic dysfunctions associated with obesity and MetS (Palhares et al., 2015; Sponchiado et al., 2016).

Ethnomedicines have therapeutic and preventive potential attributed to the presence of beneficial phytochemicals that can act on multiple biological targets thereby offering advantages over treatments with single biological targets (Liu, 2013). As stated by the World Health Organisation (WHO), about 80% of the population in developing countries depends on ethnomedicines for basic health care (World Health Organization (WHO), 2002). Interestingly, in many developed countries the use of ethnomedicines has gained popularity with 75% of the population in France, 70% in Canada, 48% in Australia, 42% in the USA and 38% in Belgium having utilised traditional medicines at least once (WHO, 2002). These plant-derived ethnomedicines in the form of concoctions, extracts, macerations and powders contain bioactive phytochemicals including among others alkaloids, flavonoids, phenols, saponins, steroids, tannins and triterpenes, (Krishnaiah, 2007). These phytochemicals exhibit health beneficial activities such as antidiabetic, antioxidant, hypocholesteraemic, hypoglycaemic, hypolipidaemic and cardioprotective (Mbikay, 2012; Razis et al., 2014; Stohs and Hartman, 2015; Subramaniam et al., 2011).

One of the plants that have been broadly used and continues to be used as ethnomedicine is *Moringa oleifera* Lam (Fadeyi et al., 2015). *M. oleifera,* commonly known as the Horseradish belongs to the Moringaceae family (Olson et al., 2016) and is a key source of naturally derived biologically active phytochemicals such as alkaloids, flavonoids, quercetin, kaempferol, tocopherols (Landrault et al., 2001), glycosides and benzyl isothiocyanate (Das and Narasimha, 1958). These bioactive phytochemicals have demonstrated numerous beneficial properties among them anti-oxidant (Siddhuraju and

Becker, 2003) and anti-hyperlipidaemic (Prasanna and Mandapaka, 2013) and hypoglycaemic (Ajibola et al., 2014). Based on the phytochemical composition and the biological activities exhibited by the phytochemicals in *M. oleifera*, extracts from the tree parts it can potentially be used to alleviate diet-induced metabolic derangements.

1.1 Justification of the study

Several studies have assessed the therapeutic potential of medicinal plants against obesity and obesity-related metabolic derangements (de Freitas Junior and de Almeida, 2017; González-Castejón and Rodriguez-Casado, 2011; Hassan and El-Gharib, 2015; Mohamed et al., 2014). However, most animal studies assessing the therapeutic potential of medicinal plants against obesity have used drug-induced obesity rat models (Chinedu et al., 2015; Gupta et al., 2012). Research making use of chemically-induced metabolic derangements do not adequately mimic diet-induced metabolic derangements brought about, for instance, by the consumption of high-fructose high-fat diets (Parasuraman et al., 2015), thus such models fail to adequately give information pertaining to metabolic problems wrought by poor dietary choices. This study made use of diet-induced metabolic disease models that closely mimic obesity and obesity-associated diseases in humans caused by poor dietary choices. Most previous and current research on the therapeutic and prophylactic potential of *M. oleifera* have employed adult and mostly only male animals (Jaiswal et al., 2013, 2009; Mehta et al., 2003; Nahar et al., 2016; Ndong et al., 2007; Oyedepo et al., 2013).

The utilisation of adult animals (rats) fails to mimic obesity in growing and adolescent children. Additionally, Mauvais-Jarvis (2015) contend that at puberty hormone-driven differences exist between male and female animals; for instance, sex-specific differences in energy homeostasis and metabolic derangements. Bridgewater et al. (2017) reported that animal studies show the existence of gender-based differences in animal models in regard to their responses to treatment interventions. Thus, focusing on male animal models does not account for such differences hence conclusions drawn regarding the potential efficacy of the material under investigation may not be true in the other sex. Studies employing the use of both sexes potentially result in the development of relevant sex-based prophylactic or therapeutic interventions that could

be utilised to mitigate obesity and its related metabolic diseases. The current study, therefore, made use of both female and male growing rats to capture on possible sex-driven differences on the potential of *M. oleifera* seed extract to prevent metabolic derangements induced by high-fructose diet. *M. oleifera* seed extract contains bioactive phytochemicals among them alkaloids, glycosides, flavonoids, phenols, saponins, tannins and phytosterols such as beta-sitosterol (Govardhan Singh et al., 2013; Vongsak et al., 2013). Flavonoids, saponins and tannins exhibit antioxidant, hypoglycaemic and hypolipidaemic properties thus the *M. oleifera* seed extract could potentially prevent fructose-induced metabolic derangements.

1.2 Aim of the study

This study evaluated the potential of crude hydroethanolic *M. oleifera* seed extract to protect against high-fructose diet-induced metabolic derangements in growing Sprague-Dawley rats modelling growing children fed high-calorie diets.

The specific objectives of the study were to:

i. qualitatively determine the presence of flavonoids, phenols, saponins, tannins, terpenoids and beta-sitosterol in hydroethanolic *M. oleifera* seed extract.
ii. determine the effects of oral administration of hydroethanolic *M. oleifera* seed extract in growing Sprague-Dawley rats fed a high-fructose diet on:
    a. body mass.
    b. tolerance to an oral glucose load.
    c. blood glucose, triglycerides and cholesterol concentration.
    d. metabolic hormone (adiponectin and insulin) concentrations.
    e. total liver lipid content.
    f. liver histology and non-alcoholic fatty liver disease activity score (NAS).
    g. surrogate marker of liver function (plasma activities aspartate transaminase and alkaline phosphatase).
    h. viscera morphometry by measuring masses of viscera.

1.3 Hypotheses

H_0: The hydroethanolic *M. oleifera* seed extract has no effect on the growth performance, ability to tolerate an oral glucose load, markers of metabolic dysfunction, viscera morphometry and surrogate markers of liver function of growing Sprague-Dawley rats fed a high-fructose diet.

H_1: The hydroethanolic *M. oleifera* affects the growth performance, ability to tolerate an oral glucose load, markers of metabolic dysfunction, viscera morphometry and surrogate markers of liver function of growing Sprague-Dawley rats fed a high-fructose diet.

The next chapter (Chapter 2) is an analysis of the literature on obesity, metabolic syndrome and consequences of metabolic syndrome such as non-alcoholic fatty liver disease (NAFLD) and type-II-diabetes mellitus. Importantly, the chapter also reviews studies on the therapeutic and prophylactic benefits derived from *M. oleifera* Lam extracts.

CHAPTER 2: LITERATURE REVIEW

2.0 Introduction

Globally communities have adopted westernised diets which are characteristically rich in calories (Carrera-Bastos et al., 2011). These diets are typically rich in processed sugars and fat (Steyn and Temple, 2012). Apart from cultural and socioeconomic and environmental factors as well as genetic predisposition, these poor dietary choices coupled with sedentary lifestyles are recognised as key mediators of obesity (Seyssel et al., 2016; Steyn and Temple, 2012) whose (obesity) prevalence is increasing globally (Ahmed et al., 2014). The observed upsurge in the prevalence of obesity is not restricted to adults; it is also true in children and adolescents (de Mello et al., 2011; Mackenbach et al., 2008; Monyeki et al., 2015). Obesity is a major health concern and due to the increase in its prevalence, it is now accorded a priority in public health policies (Hassan and El-Gharib, 2015). An estimated 1.9 billion adults worldwide were reported overweight while over 650 million were considered obese in 2016 [World Health Organisation (WHO), 2018]. While over 340 million children and adolescents were reported obese in 2016, 41 million children under the age of 5 were found to be obese (WHO, 2018). Within sub-Saharan Africa (SSA) the prevalence of obesity varies widely: from a low of 4.5% in Ethiopia to as high as 28.3% in South Africa [Central Intelligence Agency (CIA), 2016; Sartorius et al., 2015]. As reported by the South African Demographic and Health Survey (SADHS) in 2016 about 70% of South African women were obese (SADHS, 2016). Of concern in the country [South Africa (SA)] is the upsurge in the prevalence of childhood obesity. As of 2016, the SADHS survey reported childhood obesity to be 13% (SADHS, 2016). Childhood obesity increase the risk of developing NAFLD, hypertension, T-II-DM and MetS (Boden, 2011; Klop et al., 2013; Ogden et al., 2007; Promkum et al., 2010 and Roh and Jung, 2012).

2.1 Central obesity

Obesity is typified by a gain in adipose tissue and an increase in the size and number of adipocytes (Guo, 2014). While magnetic resonance imaging and computerised tomography are more precise in the assessment of central adiposity (Cristina et al., 2015), these techniques are expensive (Cristina et al., 2015). Outside of these expensive techniques, other methods and indices are used, for example, measurement of the body

mass index (BMI) (Aronne and Segal, 2002; Bays et al., 2013), waist circumference (WC) (Cristina et al., 2015; Wahba and Mak, 2007) and the waist-hip ratio (WHR) (Aronne and Segal, 2002; Bays et al., 2013). A WC greater than 88 cm and 102 cm in females and males, respectively is indicative of central obesity (Wahba and Mak, 2007). Similarly a WHR greater than 0.85 and 0.90 (for females and males, respectively) and or a BMI of 30 and above is indicative of obesity (Wahba and Mak, 2007). In children (7-9 years old) a waist to hip ratio greater than 0.5, is a predictor of amplified risk of developing metabolic diseases such as MetS in later life (Garnett et al., 2007).

2.2 Metabolic syndrome

Metabolic syndrome (MetS) affects children, adolescents and adults (Cristina et al., 2015; Friend et al., 2013). It is characterised by multiple metabolic disorders including central obesity, dyslipidaemia, hyperglycaemia, insulin resistance and hypertension (Friend et al., 2013; González-Castejón and Rodriguez-Casado, 2011; Katsumata et al., 2012; Regaieg et al., 2015). MetS intensifies the risk of developing T-II-DM, NAFLD, chronic kidney disease and cardiovascular diseases (Al-Isa, 2013; Roger et al., 2011).

To estimate the global prevalence of MetS in children and adolescents has presented to be a major challenge due to differences in definitions of and diagnostic criteria for MetS in different countries (Friend et al., 2013). While WC and WHR obesity indices have been used in assessing MetS in children, inconsistencies in cut-off points of these indices has compounded the challenge of estimating the prevalence of MetS (Friend et al., 2013). Differences in populations and age result in variations in anthropometric measurements, blood pressure and lipid profiles and thus making it difficult to use similar criteria in estimating the prevalence of MetS in children, adolescents and adults (Weiss et al., 2013). In a bid to overcome the difficulty, the International Diabetes Federation (IDF) developed a consensus definition of MetS applicable to children and adolescents (IDF, 2006; Weiss et al., 2013). The IDF proposed metabolic syndrome diagnosis in children 6-10 years as a WC $\geq 90^{th}$ percentile and the existence of risk factors inclusive of elevated triglyceride (TG) concentration, history of the cardiovascular disease in the family and hyperglycaemia (IDF, 2006; Weiss et al., 2013). The diagnosis of MetS in the 10-16 age group is premised on the presence of

hypertriglyceridemia, hypo-high-density lipoprotein(HDL)- cholesterolemia, hyperglycaemia and elevated blood pressure with a WC $\geq 90^{th}$ percentile (IDF, 2006; Weiss et al., 2013). While Friend et al. (2013) reported worldwide MetS prevalence in children and adolescents of 0 to 60% depending on diagnostic criteria used. Kaur (2014) reported a range from 10 to 84% depending on the definition used, population, age, sex and ethnicity. However, the IDF estimated the prevalence of MetS in adults to be at 25% (IDF, 2006). The treatment of metabolic syndrome includes dietary intervention, increased physical activity, lifestyle modifications, pharmacological therapies and bariatric surgery (Schauer et al., 2016).

2.2.1 Visceral obesity and metabolic syndrome

Adipocytes are involved in lipid storage, lipid synthesis and secretion of pro-inflammatory and anti-inflammatory proteins (Gustafson et al., 2009). The adipose tissue-derived adipokines adiponectin, leptin and resistin which regulate food intake and energy expenditure, inflammation, insulin secretion and sensitivity thus play a critical role in the pathogenesis of MetS (Leggio et al., 2017). Increased visceral adiposity has been observed to increase the secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Moschen et al., 2010). Tumour necrosis factor alpha inhibits lipoprotein lipase thereby stimulating lipolysis in adipocytes which elevates non-esterified fatty acids circulation (Tangvarasittichai et al., 2016). Elevated non-esterified fatty acids (NEFAs) then contribute to dyslipidaemia and insulin resistance (Chen et al., 2015). The cytokines (IL-6 and TNF-α) support the movement of macrophages to the visceral tissue which further stimulates cytokine secretion (Tangvarasittichai et al., 2016). Elevated concentrations of cytokines results in systematic inflammatory conditions which are known to cause insulin resistance (Tangvarasittichai et al., 2016).

2.2.2 Hyperglycaemia and insulin resistance

Insulin resistance is the condition whereby normal or elevated insulin concentrations result in insensitivity in target cells (Mather et al., 2013; Wilcox, 2005). This insensitivity to insulin generates compensatory hyperinsulinemia (Stumvoll et al., 2005;

Wilcox, 2005). Insulin resistance causes hyperglycaemia due to insulin not activating the signal to decrease hepatic glucose production and/or increasing muscle glucose absorption (Niswender et al., 2011). Research reported a positive association between increased adiposity with insulin resistance. Increased adipose tissue leads to upregulated releases of pro-inflammatory substances including IL-6, TNF-α, glycerol, NEFAs, leptin and resistin, (Chen et al., 2015). These adipose tissue-derived metabolites (cytokines, fatty acids, and hormones) are involved in the aetiology of insulin resistance and various other metabolic disturbances(Chen et al., 2015). Insulin stimulates fatty acid synthesis and triglyceride storage in the liver and other tissues (Wilcox, 2005). However, with insulin resistance, there is increased visceral adipose tissue lipolysis which manifests with an elevation in plasma free fatty acid, triglycerides and cholesterol concentrations. Importantly, the dyslipidaemia and insulin resistance, in turn, result in the development of T-II-DM and its sequel (Larosa et al., 2013; Matsuda and Shimomura, 2013; Reaven, 2004).

2.2.3 Dyslipidaemia

Obesity is one of the most common causes of dyslipidaemia (Klop et al., 2013). Dyslipidaemia is a condition characterised by hypertriglyceridemia, hypercholesterolemia, hyper-HDL- hypercholesterolemia and hypo-low-density lipoprotein (LDL)-cholesterolemia (Adamo et al., 2015; Farnier, 2008; Gao et al., 2014). Surplus lipid from increased central adiposity, hyperinsulinemia and or insulin resistance lead to increased availability of NEFAs and the subsequently elevated blood triglycerides (TG) concentration (Boden and Shulman, 2002). The increased blood TG concentration is subsequently followed by an increase (slight) in total cholesterol and decreased HDL-cholesterol concentration (González-Castejón and Rodriguez-Casado, 2011). In dyslipidaemia, the synthesis of TG is stimulated by esterification of glycerol phosphate while that of cholesterol is increased by activation and dephosphorylation of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (Song et al., 2017). In the liver the increased esterification of free fatty acids (FFA) stimulates the production and secretion of cholesterol, TGs and very low-density lipoprotein (VLDL) (Rutledge and Adeli, 2007). Consequently, the increase in TG concentration in hepatocytes then results in the development of NAFLD (Titmuss and Srinivasan, 2016). The increase in hepatocyte TG

concentration largely results from the imbalance between liver TG production (uptake of FFA from plasma and incorporation into VLDL and *de novo* lipogenesis) and TG removal from the liver (Fabbrini et al., 2010). Dyslipidaemia has been linked to oxidative stress which promotes the development of inflammation in blood vessels (Navarro-Millán et al., 2016). The increased availability of lipids in obesity favours lipid peroxidation, oxidation of LDL in vascular endothelium and consequently leads to plaque formation in the arteries which then increases the incidence of atherosclerosis (Matough et al., 2012).

2.2.4 Hypertension

Adiposity is one of the most common causes of atherogenic dyslipidaemia (Klop et al., 2013) which promote the development of hypertension (Leggio et al., 2017). Dyslipidaemia (abnormal blood lipid concentrations) results in the development of atherosclerosis which is a build-up of a lipid-plaque in the artery walls (Yang et al., 2017). The lipid-plaque will narrow and stiffen (reduced compliance) the artery walls via oxidative stress-induced damage thus causing hypertension (Matough et al., 2012). Adipose tissue also secretes adiponectin that has protective actions against inflammation (Ouchi and Walsh, 2007). It (adiponectin) regulates energy balance and insulin sensitivity (Leggio et al., 2017). However, this hormone is down-regulated with increased adiposity which is linked to insulin insensitivity (Leggio et al., 2017). The resultant elevation of insulin concentration then stimulates arterial dysfunction and vasoconstriction thereby promoting hypertension (Leggio et al., 2017). Apart from arterial dysfunction hyperinsulinemia also stimulate sodium reabsorption and increase sodium retention in the renal tubules (Brands and Manhiani, 2012). The hyperinsulinemic state, therefore, contributes to hypertension through volume overload resulting from increased sodium retention (González-Castejón and Rodriguez-Casado, 2011; Leggio et al., 2017). Ultimately adiposity, hyperglycaemia, insulin resistance, dyslipidaemia and hypertension will cause MetS and its associated complications (Al-Isa, 2013; Roger et al., 2011).

2.3 Complications of metabolic syndrome

Several metabolic diseases, for example, T-II-DM, NAFLD, chronic kidney disease and cardiovascular diseases manifest as complications of MetS (Al-Isa, 2013; Roger et al., 2011).

2.3.1 Type-II-diabetes mellitus

Type-II-diabetes mellitus (T-II-DM) is one of the non-communicable diseases affecting 300 million people globally (Helvaci and Ozcan, 2015). While type-I-diabetes mellitus is typified by subnormal insulin secretion by the pancreatic β-cells, T-II-DM is typified by the resistance to insulin in the peripheral tissues (Mpora et al., 2014; Rani and Srilatha, 2015). Insulin insensitivity causes the development of metabolic syndrome (MetS) which manifest with hyperglycaemia and hyperinsulinemia (Stumvoll et al., 2005). Hyperglycaemia stimulates glycation of plasma proteins inclusive of albumin, collagen, fibrinogen and globulins to various types of advanced glycation end products (AGEs) which cause diabetes-related complications (Singh et al., 2014). The hyperglycaemia and hyperinsulinemia ultimately result in the development of T-II-DM and diabetes-related complications (Chen et al., 2010; Larosa et al., 2013; Matsuda and Shimomura, 2013). On the other hand, lipotoxicity is caused by adipocytes' insensitivity to insulin. In adipocytes, insulin stimulates the uptake of NEFAs that result from TG lipolysis. Excess plasma NEFA, whose concentration is aggravated by obesity, impairs insulin secretion by pancreatic β-cells, stimulate gluconeogenesis and inhibits glycogenesis in skeletal muscle, exacerbates hyperglycaemia (Stumvoll et al., 2005). The secretion by adipose tissue of IL-6 and TNF-α pro-inflammatory cytokines contributes to insulin insensitivity by tissues thus contributing to hyperglycaemia (Stumvoll et al., 2005), a key feature of T-II-DM.

2.3.2 Kidney disease

MetS and obesity are risk factors for the development of chronic kidney disease (Gluba et al., 2013; Wahba and Mak, 2007). Gluba et al. (2013) reported that metabolic syndrome-mediated damage to the kidneys resulted in a multiplicity of manifestations including hyperfiltration, hyperfusion, glomerulosclerosis, glomerulomegaly, glomerular and tubulointerstitial injury, decreased glomerular filtration rate, glomerular

ischemia and tubular atrophy. Several mechanisms have been proposed on how obesity and metabolic syndrome mediate chronic kidney disease (Locatelli et al., 2006; Serra et al., 2008). The excessive leptin produced by obese individuals has been shown to upregulate intrarenal expression of the transforming growth factor-beta (TGF-β) which results in glomerulosclerosis (Wolf et al., 1999). In obese individuals' angiotensin-II-mediated stimulation of increased reactive oxygen species (ROS) production has been shown to result in renal microvascular injury, ischemia and tubulointerstitial damage (Kaur et al., 2017). The ROS reduce the production of the enzyme nitric oxide synthase, which is required for nitric oxide (NO) synthesis from L-arginine which (NO), is an important cellular signalling molecule, thereby contributing to endothelial injury associated with chronic kidney disease (Montezano and Touyz, 2012).

2.3.3 Non-alcoholic fatty liver disease

One of the major causes of liver failure is NAFLD (Kneeman et al., 2012). NAFLD results from increased liver lipid accretion in the absence of excessive alcohol consumption or the use of steatotic substance or medications (Buzzetti et al., 2016). Visceral obesity is a key risk factor for the development of NAFLD whose global prevalence is estimated to be 20–30% in adults and 7–37% in children (Anderson et al., 2015; Chalasani et al., 2012; Younossi et al., 2017). NAFLD is a precursor for non-alcoholic steatohepatitis (NASH) that is typified by hepatocyte damage, steatosis, inflammation and compromised liver function (Petäjä and Yki-Järvinen, 2016). Obesogenic diets rich in fructose have been identified as major drivers of the growth in the prevalence of NAFLD (Softic et al., 2016). NAFLD develops when liver triglyceride synthesis surpasses the rate of synthesis and export of VLDL triglyceride from the liver to the muscles and adipose tissue in absence of excessive alcohol consumption (Reaven, 2004; Kawano and Cohen 2013). The build-up of lipids increases the risk of developing peripheral insulin resistance and inflammation of the liver (Byrne, 2013). Excessive hepatic lipid accretion is associated with insulin resistance and glycaemic disturbances (Ma et al., 2008). Hyperinsulinemia and hepatic glucose production stimulate expression of sterol regulatory element binding protein which increases FFA production and subsequent fat deposit in the hepatocyte (Del Campo et al., 2018).

2.4 Models of metabolic diseases

Research has used rodent models of human diseases to investigate and fully understand the progression and physiological mechanism involved with different medical conditions (Rosini et al., 2012). Rodent models have been used to discover therapeutic and prophylactic interventions against diseases including obesity, T-II-DM, hypertension and MetS (Leong et al., 2015; Lutz and Woods, 2012). Broadly speaking three types of models have been used in the process of elucidating disease progression, management and treatment: genetic models, diet-induced models and chemical/drug-induced models (Panchal and Brown, 2011). Animal models of obesity used commonly include the leptin receptor-deficient *db/db* mouse, leptin-deficient *ob/ob* mouse and the Zucker rat (Lutz and Woods, 2012). Goto-Kakizaki rats have been used as diabetic models (Panchal and Brown, 2011). The *ob/ob* mice, *db/db* mice, Otsuka Long-Evans Tokushima Fatty (OLETF) rats and Zucker diabetic fatty rats have been employed in obesity and diabetic models (Panchal and Brown, 2011).

2.4.1 Genetic models

Rodents are generally the animals used as genetic models of obesity because of genetic mutations. Several models exist in which a single gene is dysfunctional or lacking, for example, OLETF rat lacks functional receptors responsible for triggering the secretion of the satiating hormone cholecystokinin (Lutz and Woods, 2012). The OLETF rats are used to interrogate energy homeostasis and analysing factors promoting the development of obesity and obesity-associated metabolic derangements (Bi and Moran, 2016). The rodent models include those that lack leptin production due to mutation or leptin resistance, for example, the Lep^{ob}/Lep^{ob} and Lep^{db}/Lep^{db} mice (Panchal and Brown, 2011). However, the use of genetic animal models for obesity studies had disadvantages due to the fact that the monogenic mutation mechanism did not mimic obesity and metabolic diseases in humans (Lutz and Woods, 2012).

2.4.2 Chemically induced models

Structural analogues of glucose such as alloxan and streptozotocin are used to induce selective damage to the pancreatic β-cells (Panchal and Brown, 2011) as a model of type-I-diabetes mellitus. Even though this type-I-diabetes mellitus resulting from

chemical induction had the rats exhibiting fatty liver and inflammation; they, however, did not gain weight, one of the manifestations of metabolic syndrome (Dheer and Bhatnagar, 2010). Therefore chemically-induced type-I-diabetes mellitus rat models fail to adequately mimic MetS in humans. However, manifestations of T-II-DM and MetS could be induced by combining low doses of streptozotocin and feeding a high-fat and or high-fructose diet (Ménard et al., 2010; Panchal and Brown, 2011), a hybrid model with a chemical and dietary component.

2.4.3 Diet-induced models

Diet-induced animal models are believed to better mimic metabolic responses caused by obesity and MetS in humans than most genetic and chemically-induced animal models (Rosini et al., 2012). Thus, diet-induced models are preferable for testing prospective therapeutics (Lutz and Woods, 2012). Wistar and Sprague-Dawley rats are commonly used in diet-induced models because they tend to exhibit many characteristics of obesity and metabolic syndrome (Marques et al., 2015) when compared to other rats strains. The consumption of obesogenic or hypercaloric diets, for example, diets rich in saturated fats, sucrose and or fructose has been reported to cause obesity in rats (Panchal et al., 2011) and to manifest components of metabolic syndrome (Lehnen et al., 2013).

2.5 Fructose and metabolic disorders

Due to the expansion in urban settlement, most societies have adopted westernised diets which are characterised by high concentrations of fructose, sucrose and saturated fat (Bray and Popkin, 2013; Le, 2010; Raatz et al., 2017). Excessive consumption of fructose is a major contributor to the increased prevalence of diet-induced metabolic disorders inclusive of obesity, MetS, NAFLD and T-II-DM (Lim et al., 2010; Nettleton et al., 2014; Raatz et al., 2017). Studies using rats fed high-fructose diets have shown an association between fructose consumption and several metabolic derangements (Ahmed et al., 2014; Das et al., 2012; Ferder et al., 2010).

Fructose, a sweet-tasting sugar exists naturally in fruits, vegetables and honey. The consumption of fructose (in moderate amounts) has been part of the human diet for ages

(Wolf et al., 2008). The global consumption of sugar [fructose, high fructose corn syrup (HFCS) and other caloric sweeteners containing fructose such as fruit juice concentrate] has increased dramatically (Bray and Popkin, 2013; Vos and Lavine, 2013). This increased consumption of these processed sugars has been demonstrated to have a positive correlation with the development and prevalence of NAFLD, obesity and MetS (Agrawal and Gomez-Pinilla, 2012; Dekker et al., 2010).

The association of fructose with the manifestation of metabolic disorder is ascribed to its metabolism that occurs in the liver (Agrawal and Gomez-Pinilla, 2012). In the liver, fructose is converted to glyceraldehyde and dihydroxyacetone phosphate by fructokinase and aldolase B (Stanhope and Havel, 2008). Unlike glucose, fructose bypassing the phosphofructokinase rate-limiting step restricts the amount of glucose that is converted into triglycerides (Beysen et al., 2018; Softic et al., 2016). Thus, a large proportion of the glyceraldehyde and dihydroxyacetone phosphate formed from fructose gets converted to triglycerides (Beysen et al., 2018; Softic et al., 2016; Sun and Empie, 2012). The triglycerides can infiltrate the hepatocytes and cause NAFLD or they can be packaged into VLDLs and transported to peripheral adipose tissues for storage (Kneeman et al., 2012). Visceral adipose tissue is regarded as the main lipid storage site and thus fructose consumption is likely to cause visceral obesity and various subsequent complications such as MetS and NAFLD (Agrawal and Gomez-Pinilla, 2012).

2.6 Interventions against metabolic diseases

Lifestyle changes such as increased physical activity and healthy dietary patterns are one of the most commonly recommended interventions against metabolic diseases (Saboya et al., 2016). Nonetheless, the main challenge with lifestyle interventions is the lack of compliance (Grave et al., 2010). Conventional pharmacological agents are available to manage metabolic disorders (Jo et al., 2014; Last et al., 2011). The management of total cholesterol and decreasing of LDL-cholesterol are primary targets in the treatment of metabolic derangements (Last et al., 2011). While conventional pharmacological cholesterol-lowering agents are effective in the management of obesity and metabolic disorders they elicit side effects (Last et al., 2011).

2.6.1 Conventional pharmacological agents

Fenofibrate an anti-hyperlipidaemic conventional pharmacological agent is used to manage metabolic syndrome (Fabbrini et al., 2010). This pharmacological agent (fenofibrate) has been shown to have several benefits: lowers blood triglyceride concentration and increases blood HDL-cholesterol (Scott et al., 2009) and lowers total cholesterol and low-density lipoproteins (Ling et al., 2013). Fenofibrate inhibits both *de novo* liver fatty acid and VLDL synthesis (Farnier, 2008) but promotes beta-oxidation of fatty acids (Farnier, 2008). The health beneficial effects of fenofibrate are exerted by activating the peroxisome proliferator-activated receptor α [(PPARα); Farnier, 2008]. PPARα, a nuclear receptor protein, functions as a transcription factor activating and regulating lipid metabolism (Dunning et al., 2014). It activates genes that regulate lipoprotein lipase which is responsible for the hydrolysis of plasma triglycerides (Kraja et al., 2010). It also regulates acyl-Co-A oxidase and carnitine palmitoyltransferase that promote β-oxidation of FFA (Kraja et al., 2010). Additionally, fenofibrate inhibits cholesterol synthesis (Caldwell, 1989; Trapani et al., 2012) and also lowers its (cholesterol) concentration in the blood by inhibiting its absorption from the small intestines (Valasek et al., 2007). Fenofibrate increases the activity of lecithin cholesterol acyltransferase (LCAT) in both normolipidaemic and hypercholesterolemic individuals (Rousset et al., 2011). The increased LCAT activity helps in the maintenance of equilibrium between the cholesterol concentration in the plasma and in the tissues (Rousset et al., 2011). While conventional pharmaceutical agents, for example fenofibrate, can be utilised in the management of metabolic derangements they are known to be relatively of a high cost, have limited accessibility and elicit side effects (Cameron et al., 2011). These challenges associated with the reliance on such conventional pharmacological agents have resulted in a shift towards more dependence on ethnomedicines; especially plant-derived ethnomedicines to manage diseases. The latter which are natural are deemed safer (Sponchiado et al., 2016). One such plant used as an ethnomedicine is *M. oleifera* which is reported to provide accessible, affordable and valuable remedies to a number of diseases (Benzie and Wachtel-Galor, 2011).

2.6.2 Ethnomedicines

Since time immemorial medicinal plants have been used to cure common ailments and to promote good health (Petrovska, 2012; Posmontier, 2011). Plant-derived

ethnomedicine and offer an alternative to conventional pharmacological agents in the management of diseases. Bitter orange, cherries, curcumin, green tea, ginger (Kazemipoor et al., 2012) and *M. oleifera* (Ahmed et al., 2014) are some of the plants that are claimed to be of help in the treatment of obesity (Ahmed et al., 2014; Bais et al., 2014). The plant-derived ethnomedicines contain phytochemicals which have beneficial properties that could be tapped in order to protect against and possibly treat obesity and its associated metabolic diseases. Among the many plants from which ethnomedicines are derived, *M. oleifera* Lam is one of the major medicinal plants (Mishra et al., 2011).

2.7 Moringa oleifera

2.7.1 Taxonomical classification

M. oleifera Lam is a deciduous plant belonging to the family Moringaceae (Mishra et al., 2011; Zaku et al., 2015).

2.7.2 Botanical description

M. oleifera is a rapidly-growing deciduous and perennial plant commonly called horseradish and or, drumstick tree (Barnes et al., 2007; Fahey, 2005). The plant grows in humid and hot arid areas (Posmontier, 2011; Toma and Deyno, 2014). The mature tree has whitish-grey bark while the immature tree's stem bark is purplish to greenish-white. The *M. oleifera* flowers which are 1-2 cm long are bisexual and have about five yellow-white petals (Posmontier, 2011). The tree's pods whose length ranges from 20-60 cm resemble thin bean or pea pods and contain 10-35 seeds. The *M. oleifera* fruit ripens about 3 months after flowering. The seed hull is semi-permeable and brownish in colour. The hull has 3 papery whitish "wings" that are at about 120-degree intervals (Posmontier, 2011). A single *M. oleifera* plant produces seeds ranging from 15 000 to 25 000 per year (Paliwal et al., 2011). The tree grows to a height of about 12m (Elangovan et al., 2015).

Figure 2.1: The leaves and flowers of *M. oleifera* grown at VOPI-ARC, South Africa (Source: Mapfumo. M, 2017).

2.7.3 General uses of M. oleifera tree

All parts of *M. oleifera* tree that is, bark, flowers, leaves, oil, roots, sap and seeds are widely utilised in traditional medicine and as food sources. *M. oleifera* leaves contain fatty acids, amino acids, vitamins and minerals and are consumed as a vegetable (Mbikay, 2012; Razis et al., 2014; Teixeira et al., 2014). *M. oleifera* seed cake is used as a fertiliser as well as a feed for livestock (Fahey, 2005). Industrially, *M. oleifera* oil is used to prepare food, perfumes and hair care products. The *M. oleifera* seeds can be consumed fresh or dried after roasting or as a tea (Fahey, 2005).

2.7.4 Moringa oleifera seed

M. oleifera seeds contain up to 40% oil. The seed oil has attracted scientific interest because of its high oleic acid (70%) content (Zaku et al., 2015). The oil, which is resistant to oxidative degradation (Fahey, 2005) is used for culinary purposes and in the industry as a lubricant, biodiesel and as a raw material in the manufacture of cosmetics (Fahey, 2005). The *M. oleifera* seed cake, a by-product from the extraction of oil is used

for the purification of water and production of organic fertilizer (Fahey, 2005; Mahajan et al., 2009).

2.7.4.1 M. oleifera seed: chemical composition

The seed contains 31.4% crude protein, 18.4% carbohydrate, 7.3% fibre and 6.2% ash (Leone et al., 2016). The *M. oleifera* oil extracted from the seeds comprises of approximately 82% unsaturated fatty acids and the dominant (70%) monounsaturated fatty acid in the seed oil is oleic acid (Zaku et al., 2015).

2.8 *M. oleifera*: phytochemical content

Various parts of *M. oleifera* contain a host of phytochemicals. The powder from the leaves of *M. oleifera* contains alkaloids, flavonoids, tannins, glycosides, phenols and saponins (Mensah et al., 2012) while its flowers have been shown to contain alkaloids, flavonoids, quercetin, glycosides, terpenoids, tannins and steroids (Alhakmani et al., 2013). The ethanolic *M. oleifera* seed extract is rich in rhamnose, glucosinates, isothiocyanates, various derivatives of β-sitosterol, niazimicin, niaziridin and niazarin (Guevara et al., 1999). Other studies also demonstrated the presence of catechin, caffeic acid, cinnamic acid, epicatechin, gallic acid, vanillin, protocatechuic acid, quercetin, phytosterols, glycosides, ferulic acid and phenols in the seed flour (Govardhan Singh et al., 2013; Hukkeri et al., 2006). While procyanidins such as 3-dibenzyl urea, aurantiamide acetate, quercetin rhamnoglucoside, quercetin glycoside and chlorogenic acid have been demonstrated to be present in the *M. oleifera* roots (Bellostas et al., 2003; Sashidhara et al., 2009), the bark contains procyanidin, tannins, moringine, alkaloids, triterpenoids, moringinine, glycosides, octacosanoic acid, sitosterol and sterols ((Bellostas et al., 2003; Kumbhare et al., 2012). The multiple phytochemicals in the various parts of *M. oleifera* have health beneficial biological activities including among others hypolipidaemic (Metwally et al., 2017), antidiabetic (Al-Malki and El Rabey, 2015), antioxidant (Alhakmani et al., 2013), anti-inflammatory (Mahajan et al., 2007) and antihypertensive (Faizi et al., 1998).

2.9 *M. oleifera*: key research findings

Several remarkable research findings have been reported with regards to the health beneficial activities of preparations from various parts of *M. oleifera.*

2.9.1 Anti-hyperlipidaemic benefits

The daily oral administration of ethanolic *M. oleifera* leaf extract at 600 mg/kg body mass for 12 weeks mitigated obesity, insulin resistance, reduced body mass and improved atherogenic index and glucose homeostasis in obese female rats (Metwally et al., 2017). *M. oleifera* leaf extract exerted its anti-obesogenic and antidiabetic by down-regulating pro-inflammatory adipokine coding mRNA gene expression and upregulating anti-inflammatory adipokine coding mRNA gene expression (Metwally et al., 2017). In obese individuals, leptin concentration is elevated but its leptin sensitivity decreases with an increase in adiposity leading to the inability of the body to detect satiety (Pan et al., 2014). This reduced sensitivity to leptin consequently leads to weight gain and hyperlipidaemia (Pan et al., 2014). In two studies, the oral administration of crude *M. oleifera* leaf extract (Ghasi et al., 2000) and ethanolic *M. oleifera* leaf extract to obese rats fed a high-cholesterol diet lowered blood cholesterol to levels comparable to the effect of simvastatin (Ahmed et al., 2014) thus demonstrating the potential of the extracts to be used as cholesterol-lowering agents. Similarly, in hyperlipidaemic rats, orally administered methanolic *M. oleifera* leaf extract at 150, 300 and 600 mg/kg for 30 days lowered the serum cholesterol, triglyceride, LDL and VLDL concentration and reduced the atherogenic index (Jain et al., 2010). These findings highlight the potential antioxidant, anti-obesogenic and anti-diabetic properties of *M. oleifera* leaf extract.

2.9.2 Antidiabetic benefits

Elevated concentrations of lipid peroxide, IL-6 and fasting blood glucose are components of the manifestation of type-II-diabetes mellitus (Al-Malki and El Rabey, 2015) and hyperglycaemia promotes the glycation of plasma proteins causing diabetic complications (Singh et al., 2014).

Orally administered *M. oleifera* seed powder at 50 and 100mg/kg body mass in streptozotocin-induced diabetic rats normalised the rats' lipid peroxide, IL-6 and fasting blood glucose concentration (Al-Malki and El Rabey, 2015) indicating the powder's

efficacy in mitigating some metabolic components of the sequelae resulting from type-II-diabetes mellitus. In alloxan-induced hyperglycaemia rats, the orally and or intraperitoneal administration of aqueous *M. oleifera* seed extract at 400 mg/kg mitigated the hyperglycaemia (Ajibola et al., 2014) thus demonstrating the *M. oleifera* seed extract's hypoglycaemic properties.

2.9.3 Antioxidant benefits

Lipid peroxidation is a free radical driven chain reaction whereby oxidative degradation of lipids (polyunsaturated fatty acids) results in cell damage (Borza et al., 2013). Aqueous *M. oleifera* leaf extract at 50 mg/mL for 1 hour showed cytoprotective properties in KB tumour line cells against hydrogen peroxide-induced cell death (Sreelatha and Padma, 2011). The protective effect of the extract is hypothesised to emanate from the antioxidant activity of the phenolic compounds which shields cells from potential oxidative damage by hydrogen peroxide (Sreelatha and Padma, 2011). In vitro the aqueous *M. oleifera* leaf extract was shown to increase the activity of catalase, glutathione peroxidase and superoxide dismutase; enzymes which constitute a battery of defence against oxidative damage to cells (Jaiswal et al., 2013; Sreelatha and Padma, 2011). Verma et al. (2009) demonstrated that orally administered methanolic *M. oleifera* leaf extract at 50 and 100 mg/kg body mass for 14 days protected rats against carbon tetrachloride-induced lipid peroxidation. *M. oleifera* possesses antioxidants (Alhakmani et al., 2013; Vongsak et al., 2013) which reduce and prevent oxidative damage of DNA (Singh et al., 2009) and that also inhibit hepatic lipid peroxidation (Verma et al., 2009).

2.9.4 Antiinflammatory benefits

Kooltheat et al. (2014) showed decreased IL-6, TNF and IL-8 production in human macrophages which were treated with ethyl acetate *M. oleifera* leaf extract. Increased cytokines (IL-6, TNF) secretion cause inflammation and tissue damage and contributes to the pathophysiology of obesity, diabetes, hypertension and atherosclerosis (Rana et al., 2007). Cheenpracha et al. (2010) and Muangnoi et al. (2012) demonstrated in lipopolysaccharide-induced murine RAW2647 macrophages that the ethanolic *M.*

oleifera pod extract inhibited nitric oxide (NO) production through its (*M. oleifera* extract) inhibitory action on the transcription of inducible nitric oxide synthase (iNOS). Inhibition of iNOS reduces the degree of inflammation (Tripathi et al., 2007) and administration of ethanolic *M. oleifera* pod extract, therefore, reduces inflammation (Muangnoi et al., 2012). Mahajan and Mehta (2010) measured delayed-type hypersensitivity reactions and humoral antibody responses in Swiss albino mice using Sheep red blood cells as the antigen. The oral administration of ethanolic *M. oleifera* inhibited type-IV T-lymphocytes and macrophage-mediated reaction resulting in reduce oedema in mice paws (Mahajan and Mehta, 2010). Administration of ethanolic *M. oleifera* seed extract to carrageen-induced oedema rats reduced serum concentration of cytokines TNF-α, IL-6, and IL-1 and subsequently reduced inflammation in the rat paws. An investigation of Wistar rats with acetic acid-induced colitis found that orally administered hydroalcoholic *M. oleifera* seed extract reduced the weight of the distal colon (Minaiyan et al., 2014). The weight of distal colon is a marker for assessing inflammation, therefore hydroalcoholic *M. oleifera* seed extract exhibits anti-inflammatory properties (Minaiyan et al., 2014).

2.9.5 Tissue protection benefits

In their investigation of the potential protective effects of aqueous *M. oleifera* seed extract in arsenic-induced hepatic toxicity in female Wistar, Chattopadhyay et al. (2011) observed that orally administered aqueous *M oleifera* seed extract (500 mg/100 g body mass/day for a period of 24 days decreased the plasma activities of alanine transaminase (ALT) and aspartate transaminase and decreased plasma cholesterol, triglycerides and LDL concentration thus demonstrating the extract's hepatoprotective and hypolipidaemic activity. In the same study, the extract was shown to reduce hepatocellular degeneration thus affirming the observed decrease in ALT and ALT activities, which are biomarkers of liver function. In mice fed a high-fat diet the oral administration of aqueous *M. oleifera* leaf extract 150mg/kg for 15 days was reported to protect against liver damage (Das et al., 2012) which was shown by a reduction in serum ALP, ALT and AST activities (Das et al., 2012). Similarly, Hamza (2010) reported that orally administered 1000 mg/kg/body mass of ethanolic *M. oleifera* seed extract exerted hepatoprotective effect in carbon tetrachloride poisoned male rats. These

results suggest that *M. oleifera* extracts could potentially be utilised as prophylaxis against liver dysfunction.

2.10. *M. oleifera*: toxicity screening

Ajibade et al. (2013) revealed that orally administering methanol *M. oleifera* seed extract at 400 and 800 mg/kg for 21 days did not affect plasma activities of aspartate transaminase and alkaline phosphatase which are liver function markers. Acute toxicity screening tests revealed that the maximum safe dose of methanolic *M. oleifera* seed extract is 3000 mg/kg (Ajibade et al., 2013). Therefore, research evidence suggests that *M. oleifera* seed extract is safe to use for medicinal purposes.

The beneficial effects of *M. oleifera* seed extract make it a feasible prophylaxis against metabolic syndrome and associated metabolic complications. The next chapter outlines the materials and methodologies used in the current study.

CHAPTER 3: MATERIALS AND METHODS

3.0 Source of *Moringa oleifera* seeds

M. oleifera seeds were sourced from the Agricultural Research Council's Vegetable and Ornamental Plant Institute (ARC - VOPI) farm situated 25 km north of Pretoria along the R573 road on GPS coordinates 25°60″S 0485and 28°35″E 9779. The area receives an average of 732mm of rainfall annually and has an average annual minimum and maximum temperatures of 11°C and 22.4°C respectively (Climate-Data.org, 2017). Specimen branches of the *M. oleifera* from ARC-VOPI (where *M. oleifera* seeds were sourced) were submitted to the University of the Witwatersrand's Herbarium for identification. Mr Donald McCallum, a botanist at the Herbarium identified and authenticated the *M. oleifera*. The collected specimen was stored at the Herbarium under reference number *Erlwanger 1.*

3.1 Preparation of the seed extract

M. oleifera seeds were manually dehulled. The dehulled seeds were then dried in an oven (Salvis ®, Salvis Lab, Rotkreuz, Schweiz, Switzerland) at 40°C for 24 hours after which they were ground to a meal using a table blender (Defy SM 720B, Shanghai, China). The resultant meal was sieved through a 1mm screen to get a fine meal. The meal was then extracted with 70% (v/v) aqueous ethanol to generate the seed extract. The extraction process was as described by Fawole et al. (2009). Briefly, 150g of the seed meal was mixed with 3000mL of 70% (v/v) aqueous ethanol in a flat-bottomed conical flask. The flask containing the mixture was then plugged with cotton wool and the contents mixed and sonicated. Following sonication for an hour, the mixture was filtered twice: firstly, using a mutton cloth and secondly through a Whatman filter paper (Whatman™, No. 4 size 150mm Ø, Hebei, China) using Millipore XX1504700 fritted base glass filtration apparatus (Millipore®, Darmstadt, Germany) under vacuum. The filtrate was then concentrated using a rotor-evaporator [Labocon (Pty) Ltd, Krugersdorp, South Africa] at 60°C for 3 hours and then dried in an oven (Salvis ®, Salvis Lab, Rotkreuz, Schweiz, Switzerland) at 40°C for 24 hours (Dangarembizi et al., 2014). Following the computation of the yield, the crude seed extract was then stored in an airtight dark glass bottle at 4°C until use.

3.2 Determination of the phytochemical composition of the *M. oleifera* seed extract

The flavonoid, saponin, tannin and terpenoid content of the *M. oleifera* were qualitatively determined as described below.

3.2.1 Determination of flavonoids

The qualitative assay for the presence of flavonoids was done as described by Ejikeme et al. (2014) with slight modification. Briefly, 1000mg crude *M. oleifera* seed extract was dissolved in 2ml of dimethyl sulfoxide. Thereafter, 2ml of the crude *M. oleifera* seed extract was then mixed with 8ml distilled water and allowed to stand for about 2 hours. To the 10 ml mixture, 5ml of ammonia solution was added. Thereafter 3ml of concentrated sulphuric acid was added to the mixture. The development of a yellow colouration which disappeared on standing confirmed the presence of flavonoids.

3.2.2 Determination of saponins

The qualitative assay for the presence of saponins was done as described by Ejikeme et al. (2014)with slight modification. Briefly, 1000mg crude *M. oleifera* seed extract was dissolved in 2ml of dimethyl sulfoxide. Thereafter, 1ml of the crude *M. oleifera* seed extract was then mixed with 4ml distilled water in a test tube. The contents were boiled in a water bath for 10 minutes. The mixture was agitated vigorously for 3 minutes to produce a stable persistent froth. The presence of saponins was confirmed by the formation of an emulsion on the addition of three drops of olive oil to the agitated mixture.

3.2.3 Determination of tannins

The qualitative assay for the presence of tannins was done as described by Ejikeme et al. (2014) with slight modification. Briefly, 1000mg crude *M. oleifera* seed extract was dissolved in 2ml of dimethyl sulfoxide. Thereafter, 1ml of the crude *M. oleifera* seed extract was then mixed with 4ml distilled water in a test tube. The contents were boiled in a water bath for 10 minutes. Thereafter 3 drops 0.1% ferric chloride was added to the boiled mixture. The development of a dark purple-black colour confirmed the presence of tannins.

3.2.4 Determination of terpenoids

The qualitative assay for the presence of terpenoids was done as described by Ejikeme et al. (2014) with slight modification. Briefly, 1000mg crude *M. oleifera* seed extract

was dissolved in 2ml of dimethyl sulfoxide. Thereafter, 1ml of the crude *M. oleifera* seed extract was then mixed with 4ml distilled water in a test tube. Thereafter 2ml chloroform and 3ml concentrated sulphuric acid were added to the mixture. The presence of terpenoids was confirmed by the formation of a reddish-brown colouration in the bottom layer of the contents in the test tube.

3.3 Ethical clearance and study site

The study, carried out at the Central Animal Services unit (CAS) and School of Physiology laboratories, was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg, South Africa; clearance certificate number 2016/10/44C.

3.4 Animals and animal management

A total of eighty-eight 21-day old Sprague-Dawley rat pups (40 males and 48 females) were used in the study. Each rat pup was individually housed in a Perspex cage with clean wood shavings for bedding. The bedding was changed twice per week. The rats were fed a standard rat chow supplied by Nutritionhub (PVT) LTD, Stellenbosch, South Africa. The nutrient content of the rat chow was as follows: crude protein 240 g/kg, crude oils and fats 53 g/kg, linoleic acid 14 g/kg, crude fibre 45 g/kg, crude ash 75 g/kg, calcium 14 g/kg, phosphorus 8 g/kg, vitamin $A_{(min)}$ 16 000 IU/kg, vitamin D 2000 IU/kg and vitamin E 100 mg/kg. Each rat had ad libitum access to the chow and either water and or 20% fructose solution as drinking fluid depending on the treatment regimen. A 12-hour light cycle was adhered to (lights on 7 am to 7 pm) and the room temperature was maintained at 25±2°C. The rats were given a 2-day habituation period prior to the commencement of the experiment.

3.5 Study design

Eighty-eight 21-day old Sprague-Dawley rat pups (40 females; 48 males) were randomly allocated to one of the following treatment regimens: group I - standard rat chow (SRC) + plain drinking water (PW) + plain gelatine cubes (PC), group II - SRC +

20% (w/v) fructose in drinking water (FW) + PC, group III - SRC + 20% FW + fenofibrate gelatine cubes (FN) at 100mg/kg/day, group IV - SRC + 20% FW + low dose of *M. oleifera* gelatine cubes (LMol) at 50mg/kg/day and group V - SRC + 20% FW + high dose of *M. oleifera* gelatine cubes (HMol) at 500mg/kg/day. The dosage of fenofibrate and that of the *M. oleifera* seed extract were as previously described by Ferreira et al. (2008) and Ajibola et al. (2014), respectively. *M. oleifera* is widely cultivated as vegetable for human consumption and as a dietary supplement (Zvinorova et al., 2015). The dosage was chosen because it was below the maximum safety dose of 3000 mg/kg (Ajibade et al., 2013). Additionally, administration of medicinal extracts at this dosage have also been proved to produce desirable therapeutic and prophylactic effects against metabolic derangements such as obesity in previous studies (Ajibola et al., 2014; Stohs et al., 2015). The gelatine cubes used as the vehicle for the administration of fenofibrate and the *M. oleifera* seed extract vehicle was prepared as described by Kamerman et al. (2004). The rats were subjected to their respective treatment regimens for 12 weeks.

3.6 Body mass measurements

The rats were weighed twice per week on an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg) to monitor growth performance and to ensure adherence to the accurate fenofibrate and *M. oleifera* seed extract dose.

3.7 Oral glucose tolerance test

After 12 weeks of treatment, on post-natal day 105 oral glucose tolerance test (OGTT) was performed. Following an overnight 12-hour fast, the basal glucose concentration of each rat was determined as described by Ghezzi et al. (2012) using a calibrated glucometer (Contour Plus, Bayer, Isando, South Africa) as per the manufacturer's instructions. Briefly, the area to be pricked was swabbed with an alcohol impregnated swab. A blood sample was then taken by pin-pricking the tip of the tail to determine basal blood glucose concentration (time = 0). Immediately after determination of the fasting basal blood glucose concentration, each rat was then gavaged via orogastric intubation with 2 g/kg body mass of sterile 50% w/v D-(+)-glucose solution [Merck

Chemicals (Pty) Ltd, Johannesburg, South Africa]. Post-gavage blood glucose concentration at time intervals 15, 30, 60 and 120 minutes was then determined as previously described.

3.8 Terminal procedures

Following the OGTT, the rats were returned to their respective treatment regimens for 48 hours to allow them to recover. Thereafter, they were fasted (PND 108) overnight and then weighed. Fasting circulating metabolites (fasting blood glucose and triglyceride concentration) were then determined from blood drawn from the tail vein of each rat. Fasting blood glucose and triglyceride concentration was determined using a calibrated Contour plus glucometer (Bayer, Isando, South Africa) and calibrated Accutrend plus GCT meter (Roche, Mannheim, Germany), respectively. The rats were then euthanised by intraperitoneal injection with an overdose of sodium pentobarbitone (Eutha-naze, Bayer, Johannesburg, South Africa) at 200 mg/kg body mass. Following euthanasia, blood was collected via cardiac puncture using 20G needles and 10ml syringes and put into heparin-coated blood collection tubes (Becton Dickinson VACUTAINER Systems Europe, Meylan Cedex, France). The heparinised blood was spun for 15 minutes at 4000 × g at 20°Cin a Sorvall RT® 6000B centrifuge (Pegasus Scientific Inc., Rockville, USA). The plasma was pipetted into microtubes and then stored at -20°C pending further assays. Immediately following blood collection, the abdomen of each rat was cut open by a midline incision and the liver and visceral fat pad were then carefully dissected out and weighed on an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). Livers were frozen and preserved at -20°C pending the determination of total liver lipid content. The mass of each visceral organ was then computed relative to body mass as described by Nunes-Souza et al. (2016) using the equation:

organ mass relative to body mass = organ mass (g) / body mass (g) x 100.

3.9 Determination of liver lipid content

The liver lipid content was determined using a Soxtec system HT 1043 Extraction Unit (Foss Analytic, HillerØd, Denmark) as described by the Association of Official Analytical Chemist (AOAC, 1995; method number 920.39). Briefly, a 3g liver sample was weighed, put into an extraction thimble and covered with absorbent cotton. Sixty (60) ml of 80% petroleum ether was added to a pre-weighed flask. The thimble was then inserted into the flask which was placed on an extraction unit. The samples were extracted for 30 minutes at 90°C. The extracted oil in the flask was dried in an oven at 110°C for 30 minutes. After drying, the flask with oil was placed in a desiccator and cooled for 30 minutes and then weighed. The lipid (oil) was weighed and the percentage ether extract was calculated using the equation:

% EE = [(weight of flask + oil - weight of flask) / initial sample weight)] x 100, where EE = ether extract.

3.10 Determination of liver micro-morphometry and NAS

The liver samples preserved in phosphate buffered formalin were routinely processed and then embedded in paraffin wax. They were then sectioned, stained with haematoxylin and eosin mounted on a glass slide, and covered with a glass coverslip. The liver histology sections were assessed and scored for ballooning, hepatic steatosis, and lobular inflammation using the semi-qualitative non-alcoholic fatty liver disease activity score system (NAS*): steatosis scoring 0: <5%; 1: 5–33%; 2: 34–66%; 3: >66%; foci of lobular inflammation scoring 0: none; 1: <2; 2: 2–4; 3: >4; hepatocellular ballooning scoring 0: none; 1: few ballooned cells; 2: many ballooned cells; total NAS (sum of values recorded for each criteria) < 2: not steatohepatitis; 3 and 4: uncertain; >5: probable or definite steatohepatitis* (Kleiner et al., 2005).

3.11 Determination of surrogate markers of liver function

The plasma activities of AST and ALP (surrogate markers of liver function), as well as the plasma concentrations of cholesterol, were measured using a colorimetric-based

clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser IDEXX Laboratories Inc., USA) according to the manufacturer's instructions.

3.12 Determination of hormone concentration

The plasma insulin and adiponectin concentration were determined using an enzyme-linked immunosorbent assay (ELISA) Rat insulin kit [Elabscience®, Rat INS (Insulin), USA] and Rat adiponectin kit [Elabscience®, Rat ADP (Adiponectin), USA], respectively. The kits were used according to the manufacturer's instructions. The absorbance values of each sample and each standard solution for the respective ELISA kit were determined using a microplate reader (Multiskan Ascent, Lab system, model nº 354, Helsinki, Finland) at 450nm wavelength. A respective standard curve for each ELISA kit was obtained by plotting the respective absorbance values against the known standard solution concentrations. The insulin and or adiponectin concentration of each plasma sample was obtained by reading off from the respective p standard curve.

3.12.1 Computation of homeostatic model assessment of insulin resistance

The HOMA-IR used for the assessment of insulin resistance was computed as described by Matthews et al. (1985) using the equation: HOMA-IR = [fasting insulin (ng/mL × fasting glucose (mg/dL)/405].

3.13 Statistical analysis

Data analysis was done using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, California, USA). The data are expressed as mean ± SD. The data for OGTT and weekly body masses within the group were analysed using repeated measures analysis of variance. Multiple-group data for other parameters were analysed by a one-way analysis of variance followed by Tukey's *post hoc* test was used to compare means. Statistical significance was considered when $p \leq 0.05$.

The next chapter presents the findings of the study.

CHAPTER 4: RESULTS

4.1 *Moringa oleifera* seed extract yield and phytochemical content

Following extraction, concentration and drying a yield of 28.9% was realised for the *M. oleifera* seed extract. Table 4.1 shows the phytochemical composition of hydroethanolic *M. oleifera* seed extract. The qualitative assays on the hydroethanolic *M. oleifera* seed extract showed that the extract contained flavonoids, saponins and terpenoids but not tannins (Table 4.1).

Table 4.1: Phytochemical (qualitative) composition of the hydroethanolic *M. oleifera* seed extract

Phytochemical	Present/Absent
Flavonoids	Present
Saponins	Present
Tannins	Absent
Terpenoids	Present

4.2 Body mass

Figure 4.1 shows the induction and terminal body masses of the female rats. While the induction and terminal body masses of female rats were similar ($p > 0.05$) the rats significantly grew ($p < 0.0001$) across treatments regimens over the experimental period (Figure 4.1).

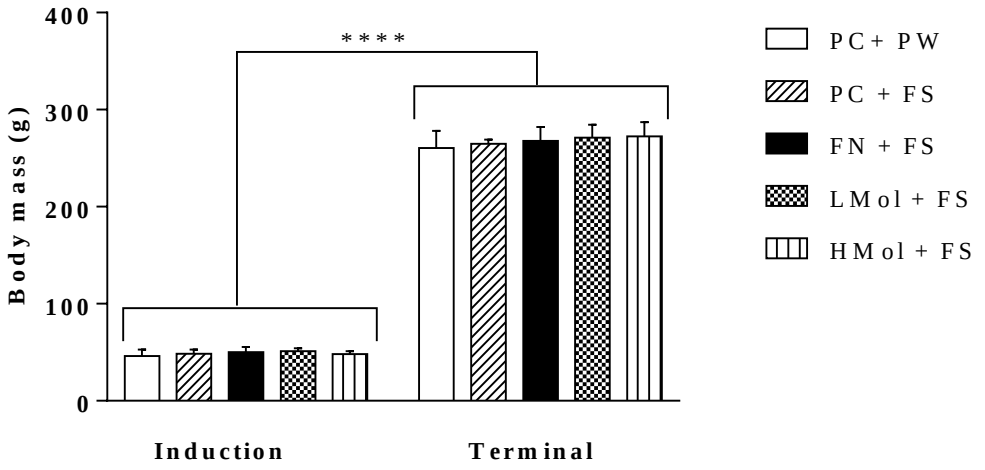


Figure 4.1: Induction and terminal body masses of female rats

**** $p < 0.0001$ when induction masses were compared to terminal masses. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution. Data presented as mean ± SD; n = 6 per treatment.

Figure 4.2 shows the induction and terminal body masses of male rats. While the induction body masses of the rats were similar, the rats grew significantly ($p < 0.0001$) during the course of the experiment. Rats that were fed the high fructose diet with fenofibrate as an intervention had significantly lower ($p < 0.01$) terminal body masses compared to that of counterparts fed the control diet (Figure 4.2).

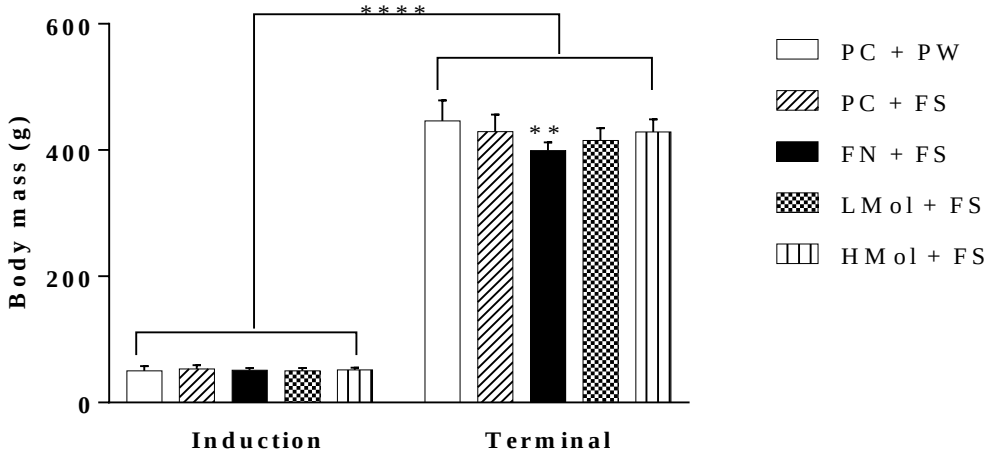


Figure 4.2: Induction and terminal body masses of male rats

**** $p < 0.0001$ when induction masses were compared to terminal masses. **$p < 0.01$ when terminal body masses of the FN + FS rats were compared to that of control (PC + PW) counterparts. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution. Data presented as mean ± SD; n = 6 per treatment.

4.3 Area under the curve of oral glucose tolerance test, glucose, triglyceride, cholesterol, insulin, adiponectin concentration and HOMA-IR

Table 4.2 below shows the AUC of OGTT, fasting glucose, triglyceride, cholesterol, insulin and adiponectin concentration and HOMA-IR of female (A) and male (B) rats. Female rats that received high-fructose diet alone or with fenofibrate had significantly increased plasma insulin and HOMA-IR ($p < 0.05$; PW + FS and FN + FS is compared to other treatments; Table 4.2A). The high-fructose diet increased ($p < 0.05$) plasma triglyceride concentration in both female and male rats and plasma cholesterol concentration in male rats only (Table 4.2A; 4.2B). Fenofibrate did not protect ($p > 0.05$) the female rats against the high-fructose diet-induced hyperinsulinemia and insulin resistance (Table 4.2A). The high-dose of *M. oleifera* seed extract prevented ($p > 0.05$, PC + PW is compared to HMol + FS) the high-fructose diet-induced hypertriglyceridemia in female rats (Table 4.2A). The treatments had no effect on the plasma cholesterol concentration of female rats (Table 4.2A). The high-dose

of *M. oleifera* seed extract prevented ($p > 0.05$, when PC + PW was compared to HMol + FS) the high-fructose diet-induced hypertriglyceridemia in male rats (Table 4.2B). In male rats that were administered the high-fructose diet (PC + FS) plasma cholesterol concentration was significantly increased compared to rats that received the high-fructose diet in combination with fenofibrate (FN + FS) or the low and high dose of *M. oleifera* seed extract (LMol + FS and HMol + FS; Table 4.2B). Both male and female rats had no significant differences in adiponectin concentration across treatments (Table 4.2A and 4.2B).

Table 4.2: Effect of hydroethanolic *M. oleifera* seed extract on blood parameters of female (A) and male (B) rats fed a high-fructose diet

A

	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
AUC of OGTT (mmol/L/min)	701.00 ± 63.64[a]	724.50 ± 80.99[a]	797.50 ± 56.10[a]	716.30 ± 32.14[a]	749.80 ± 49.73[a]	ns
Glucose (mmol/L)	3.90 ± 0.49[a]	4.17 ± 0.54[a]	4.30 ± 0.30[a]	3.55 ± 0.52[a]	3.67 ± 0.38[a]	ns
Insulin (ng/mL)	15.84 ± 3.73[a]	25.56 ± 5.13[b]	28.33 ± 5.28[b]	19.04 ± 5.87[a]	17.20 ± 6.78[a]	*
HOMA-IR	2.76 ± 0.75[a]	4.72 ± 0.99[b]	5.40 ± 0.93[b]	3.03 ± 1.07[a]	2.83 ± 1.21[a]	*
Adiponectin (pg/mL)	76.12 ± 40.71[a]	101.40 ± 43.47[a]	104.90 ± 47.10[a]	66.44 ± 34.98[a]	72.58 ± 49.68[a]	ns
Triglycerides (mmol/L)	1.39 ± 0.05[a]	2.36 ± 0.51[b]	2.75 ± 0.53[b]	2.15 ± 0.21[bc]	1.66 ± 0.18[ac]	*
Cholesterol (mmol/L)	1.91 ±0.15[a]	2.23 ± 0.56[a]	1.68 ± 0.39[a]	1.982 ± 0.28[a]	1.80 ± 0.25[a]	ns

B

	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
AUC of OGTT (mmol/L/min)	692.70 ± 32.71[a]	731.00 ± 34.81[a]	830.00 ± 31.34[b]	709.90 ± 32.86[a]	693.00 ± 56.32[a]	**
Glucose (mmol/L)	4.07 ± 0.21[a]	3.85 ± 0.29[a]	4.83 ± 0.21[b]	3.90 ± 0.31[a]	3.82 ± 0.33[a]	***
Insulin (ng/ml)	18.92 ± 5.97[a]	24.56 ± 7.31[a]	17.68 ± 6.19[a]	25.15 ± 8.36[a]	21.80 ± 6.78[a]	ns
HOMA-IR	3.41 ± 1.06[a]	4.26 ± 1.47[a]	3.81 ± 1.37[a]	4.32 ± 1.36[a]	3.70 ± 1.21[a]	ns
Adiponectin (pg/mL)	62.14 ± 48.88[a]	79.40 ± 61.24[a]	64.91 ± 26.89[a]	80.76 ± 46.87[a]	98.14 ± 53.37[a]	ns
Triglycerides (mmol/L)	1.51 ± 0.12[a]	2.01 ± 0.21[b]	2.16 ± 0.44[b]	2.12 ± 0.22[b]	1.63 ±0.12[a]	*
Cholesterol (mmol/L)	1.51 ± 0.16[a]	1.92 ± 0.29[a]	1.35 ± 0.35[b]	1.36 ± 0.23[b]	1.21 ± 0.28[b]	*

Different letters (ab) within rows are significantly different at $p < 0.05$ by Tukey's multiple-comparisons *post hoc* test. ns - no significant difference, $p > 0.05$. **A**: * $p < 0.05$ when PC + FS and FN + FS female rats' triglycerides, insulin, HOMA-IR were compared to that of female rats from all other treatments. * $p < 0.05$ when LMol + FS female rats' triglycerides were compared to the control female rats. **B**: * $p < 0.05$ when PC + FS, FN + FS and LMol + FS male rats triglycerides were compared to PC + PW and HMol + FS male rats. ** $p < 0.01$; ***$p < 0.001$

when FN + FS male rats AUC of OGTT and glucose concentration were compared to that of male rats from all other treatments. * $p < 0.05$ when PC + FS, FN + FS and LMol + FS male rats triglycerides were compared to PC + PW and HMol + FS male rats. * $p < 0.05$ when FN + FS, LMol + FS and HMol + FS male rats cholesterol was compared to their counterpart PC + PW and PC + FS male rats. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; AUC = area under the curve; HOMA-IR = Homeostatic Model Assessment of Insulin Resistance. Data presented as mean ± SD; n = 6 per treatment.

4.4 Viscera morphometry

The absolute and relative [to body mass (%BM)] masses of viscera of female (A) and male (B) rats are shown in Table 4.3. The high-fructose diet (PC + FS) significantly increased ($p < 0.001$) visceral fat pad masses (absolute and relative to body mass) of female rats (Table 4.3A). While the fenofibrate (FN + FS) prevented the fructose-diet induced visceral adiposity in female rats (Table 4.3A), the crude hydroethanolic *M. oleifera* seed extract (LMol + FS and HMol + FS) failed to protect against the fructose-diet induced visceral adiposity ($p > 0.05$; LMol + FS and HMol + FS compared to PC + FS) in female rats (Table 4.3A). Fenofibrate (FN + FS) significantly increased ($p < 0.001$ and $p < 0.0001$ respectively) absolute and relative to body mass liver masses in female and male rats (Table 4.3A and 4.3B).

Table 4.3: Effect of hydroethanolic *M. oleifera* seed extract on viscera morphometry of female (A) and male (B) rats fed high-fructose diet

A

Organ	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
Liver (g)	7.22 ± 0.74^{a}	8.55 ± 0.94^{a}	11.08 ± 1.84^{b}	8.33 ± 0.86^{a}	8.43 ± 0.99^{a}	***
Liver (%BM)	2.77 ± 0.25^{a}	3.23 ± 0.35^{a}	4.12 ± 0.53^{b}	3.08 ± 0.29^{a}	3.09 ± 0.25^{a}	***
Visceral fat pad (g)	9.16 ± 1.93^{a}	15.27 ± 2.43^{b}	10.82 ± 2.28^{a}	15.20 ± 3.65^{b}	13.64 ± 1.71^{b}	*
Visceral fat pad (%BM)	3.50 ± 0.58^{a}	5.77 ± 0.90^{b}	4.03 ± 0.77^{a}	5.59 ± 1.14^{b}	4.99 ± 0.48^{b}	**

B

Organ	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
Liver (g)	12.54 ± 0.71^{a}	12.65 ± 1.00^{a}	17.67 ± 1.58^{b}	12.55 ± 0.55^{a}	12.53 ± 0.91^{a}	****
Liver (%BM)	2.82 ± 0.23^{a}	2.94 ± 0.10^{a}	4.42 ± 0.33^{b}	3.03 ± 0.14^{a}	2.92 ± 0.15^{a}	****
Visceral fat pad (g)	10.57 ± 2.82^{a}	12.89 ± 2.91^{a}	11.33 ± 3.44^{a}	13.00 ± 2.08^{a}	12.20 ± 3.17^{a}	ns
Visceral fat pad (%BM)	2.35 ± 0.23^{a}	3.00 ± 0.67^{a}	2.82 ± 0.80^{a}	3.13 ± 0.45^{a}	2.84 ± 0.67^{a}	ns

Different letters (ab) within rows are significantly different at $p < 0.05$ by Tukey's multiple-comparisons *post hoc* test. ns - no significant difference, $p > 0.05$ **A**: *** $p < 0.001$ when FN + FS female rats liver masses (absolute and relative to body) were compared to that of female rats from all other treatments. * $p < 0.05$; ** $p < 0.01$ when PC + FS, LMol + FS and HMol + FS female rats' visceral fat pad masses (absolute and relative to body respectively) were compared to PC + PW and FN + FS female rats. **B**: **** $p < 0.0001$ when FN + FS male rat liver masses (absolute and relative to body) were compared to that of male rats from all other treatments. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; BM = Body mass. Data presented as mean ± SD; n = 6-10 per treatment.

4.5 Total liver lipid content

Figure 4.3 shows the liver lipid content of female (A) and male (B) rats. High-fructose diet significantly increased ($p < 0.01$; PC + FS, LMol + FS and HMol + FS are compared to control) liver lipid accumulation in both female and male rats (Figure 4.3A and 4.2B). Fenofibrate prevented ($p < 0.0001$) liver lipid accumulation in high-fructose diet-fed female rats (Figure 4.3A) In male rats, the fenofibrate and low dose of crude hydroethanolic *M. oleifera* seed extract did not prevent lipid accumulation (Figure 4.3B). However, the high dose of the crude hydroethanolic *M. oleifera* seed extract prevented ($p > 0.05$ HMol + FS compared to (PC + PW) control) the high-fructose diet-induced liver lipid accumulation in male rats but not in female rats (Figure 4.3A and 4.3B).

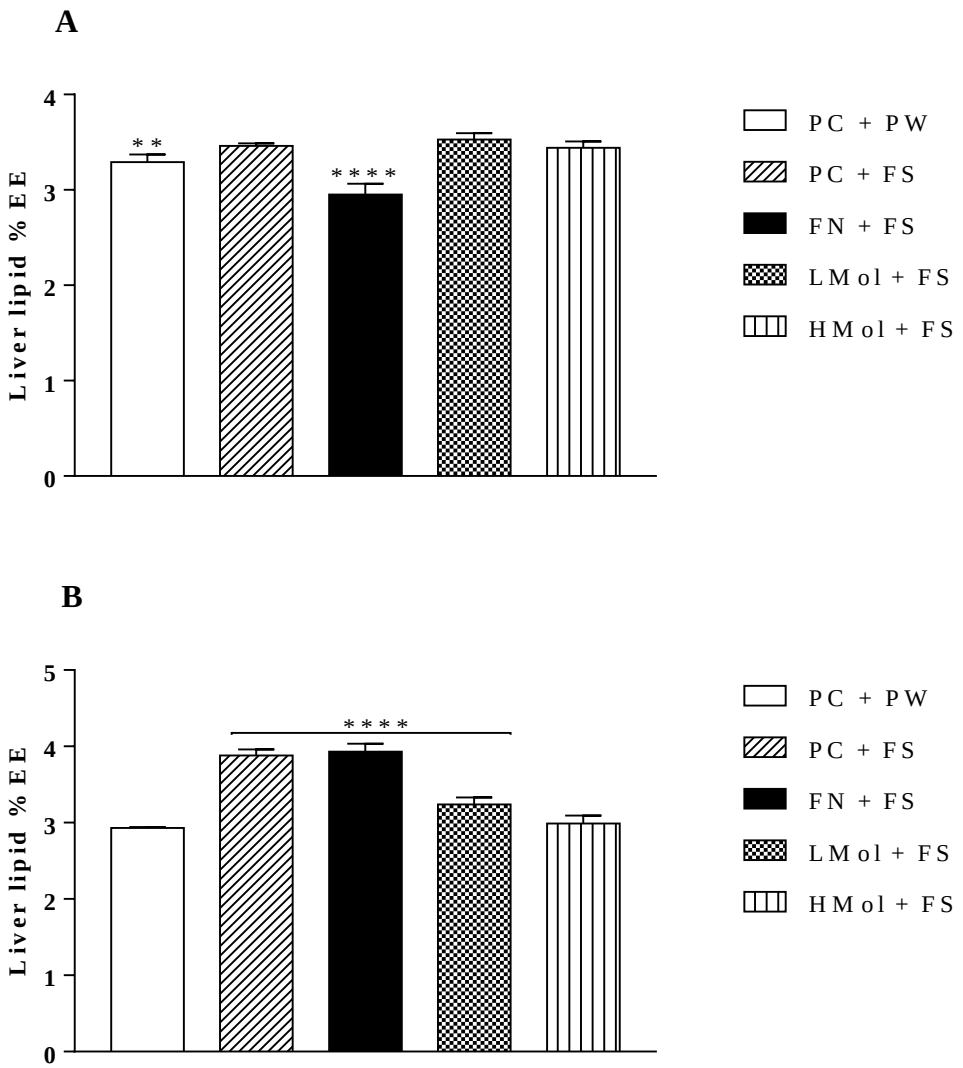


Figure 4.3: Effect of hydroethanolic *M. oleifera* seed extract on liver lipid content of female (A) and male (B) rats fed high-fructose diet

A: **p < 0.01 when PC + PW female rats liver lipid content compared PC+ FS, LMol + FS and HMol + FS rats. ****p < 0.0001 when FN + FS female rats liver lipid content compared to all other treatments. **B**: ****p < 0.0001 when PC + FS, FN + FS and LMol + FS male rats liver lipid content compared to their counterpart PC + PW and HMol + FS male rats. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; EE = Ether Extract. Data presented as mean ± SD; n = 8-10 per treatment.

4.6 Surrogate markers liver function

Table 4.4 shows plasma aspartate transaminase and alkaline phosphatase activities of female (A) and male (B) rats. Fenofibrate significantly increased ($p < 0.0001$; FN + FS is compared to other treatments) plasma alkaline phosphatase activity in male rats only (Table 4.4B). The high-fructose diet and crude hydroethanolic *M. oleifera* seed extract (low and high dose) had no effect on plasma aspartate transaminase and alkaline phosphatase activities of both female and male rats (Table 4.4A and 4.4B).

Table 4.4: Effect of hydroethanolic *M. oleifera* seed extract on aspartate transaminase and alkaline phosphatase activities of female (A) and male (B) rats fed a high-fructose diet

A

Parameter	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
Aspartate transaminase (U/L)	114.00 ± 19.49[a]	94.60 ± 27.87[a]	112.40 ± 39.26[a]	102.00 ± 38.52[a]	101.30 ± 30.92[a]	ns
Alkaline phosphatase (U/L)	122.00 ± 29.39[a]	110.70 ± 34.73[a]	162.20 ± 42.32[a]	144.60 ± 36.02[a]	113.70 ± 19.52[a]	ns

B

Parameter	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
Aspartate transaminase (U/L)	154.80 ± 64.35[a]	116.00 ± 35.41[a]	175.60 ± 38.61[a]	144.20 ± 46.25[a]	174.60 ± 56.60[a]	ns
Alkaline phosphatase (U/L)	166.10 ± 40.96[a]	163.40 ± 18.87[a]	281.70 ± 40.86[b]	180.60 ± 12.47[a]	152.70 ± 22.96[a]	****

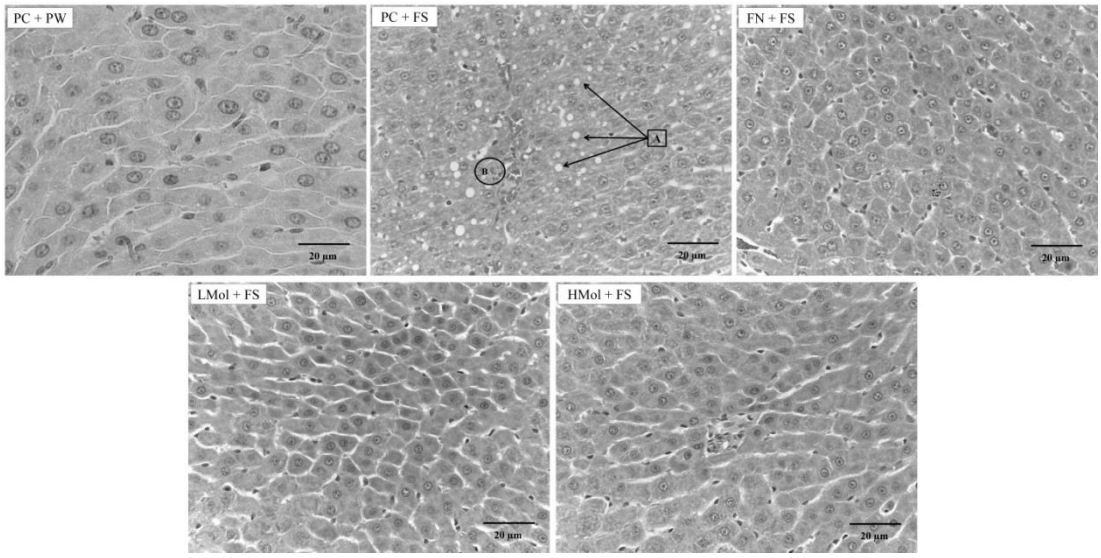
Different letters (ab) within rows are significantly different at $p < 0.05$ by Tukey's multiple-comparisons *post hoc* test. ns - no significant difference, $p > 0.05$. **A**: $p > 0.05$ when aspartate transaminase and alkaline phosphatase activities compared across treatments. **B**: **** $p < 0.0001$ when fenofibrate (FN + FS) alkaline phosphatase activity was compared to all other treatments. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution. Data presented as mean ± SD; n = 6 per treatment.

4.7 Liver histology and NAS

Figure 4.4 depicts the representative liver histology photo sections (H and E staining, 400 X magnification) of female (A) and male (B) rats. Steatosis was observed in the liver histology sections of female and male rats that received the high-fructose diet (PC + FS) without intervention (Figure 4.4). However, in female and male rats that received the high-fructose diet with fenofibrate or crude hydroethanolic *M. oleifera* seed extract (FN + FS, LMol + FS and HMol + FS) steatosis was not present (Figure 4.4). This indicates that the fenofibrate or crude hydroethanolic *M. oleifera* seed extract (low and high dose) prevented high-fructose diet-induced steatosis.

Table 4.5 represents the steatosis, lobular inflammation, ballooning and total non-alcoholic fatty liver disease activity score (NAS) of female (A) and male (B) rats. Steatosis score was significantly increased ($p = 0.03$; PC + FS is compared to other treatments) in female rats that received a high-fructose diet (Table 4.5A). The fenofibrate and crude hydroethanolic *M. oleifera* seed extract (low and high dose) prevented in female rats the high-fructose diet-induced increase in steatosis score (Table 4.5A). The total NAS was significantly increased ($p = 0.04$; PC + FS is compared to PC + PW and HMol + FS) in female rats that received high-fructose diet (Table 4.5A). In male rats, the high-fructose diet significantly increased ($p < 0.03$; PC + FS is compared to other treatments) lobular inflammation score (Table 4.5B). The fenofibrate and *M. oleifera* seed extract (low and high dose) protected ($p > 0.05$; control is compared to FN + FS, LMol +FS and HMol + FS) the male rats against the high-fructose diet-induced lobular inflammation (Table 4.5B).

A



B

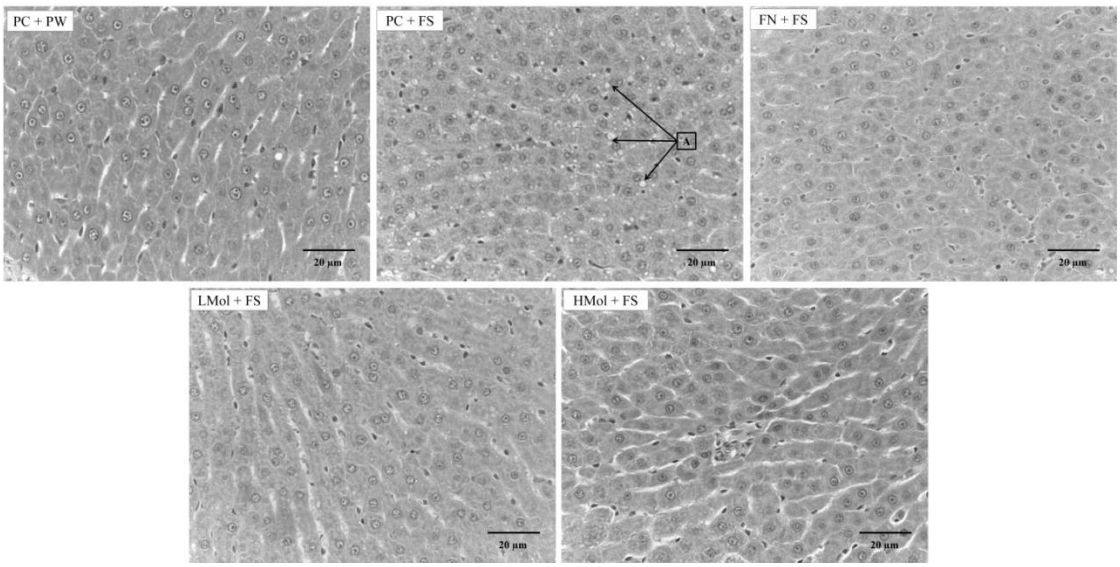


Figure 4.4: Representative liver histology photograph sections (H and E staining, 400 X magnification) of female (A) and male (B) rats following 12 weeks on respective treatments. Arrows A point to hepatic microvesicular steatosis and circle B shows foci of lobular inflammation. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution.

Table 4.5: Effects of crude hydroethanolic *M. oleifera* seed extract on non-alcoholic fatty liver disease activity score (NAS) of female (A) and male (B) rats.

A

Parameter	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
Steatosis score	0 (0, 0)[a]	2 (1, 2)[b]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	***
Ballooning score	0 (0, 0)[a]	1 (0, 1)[a]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	ns
Lobular inflammation score	0 (0, 0)[a]	1 (0, 2)[a]	1 (1, 1)[a]	0 (0, 1)[a]	0 (0, 0)[a]	ns
Total NAS	0 (0,0)[a]	3 (3, 4)[b#]	0 (0, 1)[ab]	0 (0, 1)[ab]	0 (0, 0)[a]	**

B

Parameter	PC + PW	PC + FS	FN + FS	LMol + FS	HMol + FS	Significance
Steatosis score	0 (0, 0)[a]	1 (0,1)[a]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	ns
Ballooning score	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	ns
Lobular inflammation score	0 (0, 0)[a]	1 (1, 2)[b*]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	***
Total NAS	0 (0,0)[a]	2 (2, 2)[b*]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	***

# $p < 0.05$ vs. PC + PW and HMol + PW. Different letters (ab) within rows are significantly different at $p < 0.05$ by Dunn's multiple-comparisons *post hoc* test. ns - No significant difference, $p > 0.05$. **A**: *** $p < 0.0001$ when PC + FS female rats' steatosis score were compared to that of female rats from all other treatments. ** $p < 0.01$ when PC + FS female rats' total NAS was compared to that PC + PW and HMol + FS female rats. **B**: *** $p < 0.0001$ when PC + FS male rats' lobular inflammation score and total NAS were compared to that of PC + PW and HMol + FS male rats. ** $p < 0.01$; ***$p < 0.001$ when FN + FS male rats AUC of OGTT and glucose concentration respectively were compared to male rats from all other treatments. PC + PW = standard rat chow (SRC) + plain gelatine cube + plain water; PC + FS = SRC + plain gelatine cube + 20% w/v fructose solution; FN + FS = SRC + 100 mg/kg body mass/day fenofibrate + 20% fructose solution; LMol + FS = SRC + 50 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; HMol + FS = SRC + 500 mg/kg body mass/day *M. oleifera* seed extract + 20% fructose solution; NAS = non-alcoholic fatty liver disease activity score. * $p < 0.05$ vs. all other treatments. Data presented as median and range; n = 3 per treatment.

CHAPTER 5: DISCUSSION

This discussion discourses on the phytochemical composition of crude hydroethanolic *M. oleifera* seed extract; the extract's effects on body mass, tolerance to an oral glucose challenge and sensitivity to insulin, metabolic substrate content, hepatic lipid content, liver function and viscera morphometry of growing Sprague-Dawley rats fed high-fructose diet.

In my study, qualitative assays demonstrated the presence of flavonoids, saponins and terpenoids in the hydroethanolic *M. oleifera* seed extract (Table 4.1). My finding mirrors the results reported by Idris et al. (2016) and Leone et al. (2016) of ethanolic *M. oleifera* seed extracts containing alkaloids, flavonoids, phenols, saponins and sterols (Idris et al., 2016; Leone et al., 2016). Importantly, the above-mentioned phytochemicals exert anti-inflammatory, anti-lipogenic and anti-oxidant activities (Idris et al., 2016; Leone et al., 2016). The findings of this study are therefore consistent with prior studies (Idris et al., 2016; Leone et al., 2016). Marrelli et al. (2016) reported that saponins from different natural extracts possessed anti-obesogenic properties. Similarly, saponin-enriched sea cucumber extract exhibited an anti-obesity effect through inhibition of pancreatic lipase activity in mice fed a high-fat diet (Guo et al., 2016). Based on the review by Marrelli et al. (2016) and findings by Guo et al. (2016), I speculate that saponins in the *M. oleifera* seed extract could have caused the anti-obesogenic effects exhibited in this study. Lin et al. (2018) reviewed the flavonoids in *M. oleifera* and the most common flavonoids kaempferol and quercetin have been reported to possess anti-inflammatory, anti-oxidant and hypolipidemic properties. Based on this evidence I therefore speculate that the flavonoids in the *M. oleifera* seed extract had hepatoprotective and anti-obesity effects. A review by Nazaruk and Borzym-Kluczyk (2015) suggests that triterpenoids possess antidiabetic activities through different mechanisms such as α-glucosidase and α-amylase inhibitors, aldose reductase inhibition and or as protein tyrosine phosphate 1B inhibitors among others. Therefore, the terpenoids in *M. oleifera* seed extract could have caused the antidiabetic effects exhibited in the present study. Although in the current study only qualitative assays for the phytochemical composition of the seed extract were done, the reported array of the phytochemicals noted suggests that the extract might possess, antidiabetic, anti-inflammatory, anti-obesogenic and anti-oxidant, activities. Based on the qualitative composition of the hydroethanolic *M. oleifera* seed extract, I speculated that the extract may prevent fructose-induced metabolic derangements.

Research findings on how high-fructose diets affect body mass are inconsistent. While some studies report that high-fructose diets do not cause an increase in body mass (Jatkar et al., 2017), others report that it increases body mass (Wooten et al., 2016). Consistent with the findings by Jatkar et al. (2017), the present study found that the fructose-diet had no impact on the body mass of the rats (Figure 4.1 and 4.2). In several studies, mine included, where fructose-based diets were used feed intake was not measured. Perhaps the dichotomy in the effects of dietary fructose on body mass could be due to caloric compensation with the fructose fed rats consuming less feed. There is also the possibility that since growing rats were used, the "extra" calories might have been spent supporting lean growth. My findings show that administering fenofibrate to high-fructose diet-fed male rats resulted in the reduction of terminal body masses (Figure 4.2). This finding suggests that fenofibrate may compromise mass gain in the male rats. Importantly, the crude hydroethanolic *M. oleifera* seed extracts exhibited no effect on the terminal body mass of the high-fructose diet-fed rats, thus suggesting it can be used as medicine without body mass gain problems.

In the liver, metabolism of fructose does not involve the key rate limiting step of the glycolysis pathway (Rutledge and Adeli, 2007). Consequentially, the fructose metabolism triggers increased *de novo* lipogenesis, which can lead to the excessive infiltration and accumulation of liver lipids and dyslipidaemia (Softic et al., 2016). Excessive accumulation of liver lipids could affect liver mass as well as lead to NAFLD (Beysen et al., 2018). In my present study the high-fructose diet did not affect the liver mass of the rats, it did, however, lead to increased accumulation of liver lipid and the development of steatosis in the rats (Figure 4.3A and 4.3B; Figure 4.5A and 4.5B). My present findings are consistent with previous studies where high-fructose diets were shown to cause excessive liver lipids accretion and steatosis (Choi et al., 2017; Roglans, 2002). In female rats, fenofibrate prevented excessive liver lipid accumulation brought about by the high-fructose diet (Figure 4.3A). However, in high-fructose diet-fed male rats the high dose of the crude hydroethanolic *M. oleifera* seed extract prevented the increase in liver lipid accretion (Figure 4.3B). It is speculated that fenofibrate prevented the fructose-induced liver lipid accretion in female rats by activating peroxisome proliferator-activated receptor α (PPARα) that mediate liver lipid oxidation (Ferreira et al., 2008). Both the crude hydroethanolic *M. oleifera* seed extract and fenofibrate protected against steatosis in fructose-fed rats (Figure 4.5A and 4.5B). Crude hydroethanolic *M. oleifera* seed extract contains phytochemicals which exert anti-

inflammatory, anti-lipogenic and anti-oxidant activities (Idris et al., 2016; Leone et al., 2016). I speculate that the phytochemicals in the crude hydroethanolic *M. oleifera* seed extract protected against the fructose-induced excessive liver lipid accumulation and steatosis by suppressing hepatic lipogenesis.

The NAS is used to determine severity and progression of NAFLD (Kleiner et al., 2005). Female rats fed the high-fructose diet had the most severe steatosis (Table 4.5A). I observed lobular inflammation in the liver histology sections of male rats fed a diet rich in fructose but not in those of the female rats fed the same diet. The total NAS of the rats was less than five (Table 4.5A and 4.5B) suggesting that the fructose-rich diet did not result in NASH. It could be speculated that male rats could be more prone to diet-induced hepatic inflammation. The crude hydroethanolic *M. oleifera* seed extract and fenofibrate protected against the fructose-induced hepatic inflammation. Similarly, Hamza (2010) reported that orally administered 1000 mg/kg/body mass of 70% (v/v) ethanolic *M. oleifera* seed extract exerted hepatoprotective effect in carbon tetrachloride poisoned male rats. Furthermore, my findings are consistent with the study by Halaby et al. (2015) who demonstrated that the roots and leaves of *M. oleifera* attenuated high-fat diet-induced NAFLD (Halaby et al., 2015).

The high-fructose diet resulted in hypertriglyceridemia but not hypercholesterolemia in the rats (Table 4.2A and 4.2B). Orally administered high dose crude hydroethanolic *M. oleifera* seed extract prevented fructose-induced hypertriglyceridemia in the rats (Table 4.2A and 4.2B). My findings are consistent with those by Al-Malki and El Rabey (2015) and Randriamboavonjy et al. (2016) who demonstrated that orally administered *M. oleifera* seed powder protected against dyslipidaemia in spontaneously hypertensive rats and high-fructose high-cholesterol diet-fed rats (Al-Malki and El Rabey, 2015; Randriamboavonjy et al., 2016). Randriamboavonjy et al. (2016) revealed that *M. oleifera* seed powder activates PPARs and PPAR activating prostacyclin leading to increased lipid oxidation. I therefore, speculate that the crude hydroethanolic *M. oleifera* seed extract protected against fructose-induced hypertriglyceridemia through activation of PPARs and PPAR activating prostacyclin. The anti-hyperlipidaemic activities of my crude hydroethanolic *M. oleifera* seed extract could be ascribed to the presence of flavonoids, saponins and terpenoids (Leone et al., 2016). Fenofibrate did not protect against the diet-induced hypertriglyceridemia in the rats (Table 4.2A and 4.2B). Hypertriglyceridemia augments the risk for the development of

atherosclerosis and T-II-DM (Grundy, 1999; Li et al., 2014). The female rats fed a fructose-diet developed insulin resistance and hyperinsulinemia (Table 4.2A and 4.2B) suggesting that they could be at a greater risk of developing diet-induced MetS and atherosclerosis compared to their male counterparts. Neither the fructose nor crude hydroethanolic *M. oleifera* seed extract affected the total cholesterol of the rats (Table 4.2A and 4.2B). On the other hand, fenofibrate reduced total cholesterol in male rats fed a fructose-diet (Table 4.2B).

The glucose tolerance test and its area under the curve are used to assess glycaemic control (Sakaguchi et al., 2016). In this study, the fructose-diet did not affect glycaemic control in the rats (Table 4.2A and 4.2B). My findings are at variance with those by Kosuru and Singh (2017) who reported that consumption of fructose solution caused glycaemic disturbances in adult male rats. This variance could be ascribed to the difference in the younger age of the rats. *M. oleifera* seed powder and crude aqueous *M. oleifera* seed extract have been demonstrated to exert anti-hyperglycaemic activities in streptozotocin- and alloxan-diabetic rats, respectively (Ajibola et al., 2014; Al-Malki and El Rabey, 2015). In this study the crude hydroethanolic *M. oleifera* seed extract did not affect glycaemic control in the rats (Table 4.2A and 4.2B). Interestingly, orally administered fenofibrate resulted in hyperglycaemia and glycaemic disturbances in the male rats (Table 4.2B). Similarly, Liu et al. (2011) found that orally administering 100 mg/kg fenofibrate to male rats impaired β-cell function and glycaemic control.

In the liver, the metabolism of fructose triggers increased *de novo* triglyceride (TG) synthesis (Rutledge and Adeli, 2007). The TGs are enveloped into very low-density lipoproteins (VLDL) and transported via circulation to peripheral adipose tissue (Rutledge and Adeli, 2007). Visceral adipose tissue is one of the main metabolic 'sink-areas' that is responsible for taking up and storing lipids (Dekker et al., 2010). Thus, high-fructose diets increase the availability of TGs synthesised in the liver which are exported into the periphery thus causing visceral adiposity (Rutledge and Adeli, 2007). The fructose-diet caused visceral obesity in female rats and not in males (Table 4.3A and 4.3B). My results suggest that in female rats the fructose-diet stimulated an increased liver *de novo* TG synthesis which were then shuttled to visceral adipose tissue and consequently caused visceral obesity. In female rats, fenofibrate mitigated the diet-induced visceral adiposity (Table 4.3A). Despite the crude hydroethanolic

M. oleifera seed extract exerting anti-hyperlipidaemic activities (Table 4.2A), it did not protect the female rats against diet-induced visceral adiposity (Table 4.3A). Crude hydroethanolic *M. oleifera* seed extract and fenofibrate did not affect visceral adiposity in male rats (Table 4.3B).

Adiponectin, an anti-inflammatory signalling-protein secreted by adipose tissue, increases fatty acid β-oxidation as well as the sensitivity of tissues to insulin (Kadowaki et al., 2006). Visceral adiposity is associated with hypoadiponectinemia and pro-inflammatory status (Kadowaki et al., 2006). In my present study the high-fructose diet caused obesity, however, this did not translate into hypoadiponectinemia in the rats (Table 4.2A and 4.2B). My results are challenging to explain at the moment however, one can speculate that hypoadiponectinemia did not manifest due to the rats not being in a pro-inflammatory state. Research suggests that hypoadiponectinemia manifests when the visceral adiposity is accompanied by mild to severe pro-inflammatory status that is characterised by elevated IL-6, TNF-α and C-reactive proteins (Kadowaki et al., 2006). Crude hydroethanolic *M. oleifera* seed extract and fenofibrate did not affect plasma adiponectin concentration of high-fructose diet-fed rats.

Increased plasma aspartate transaminase activity indicates hepatocyte damage (Thulin et al., 2008), whereas, increased plasma alkaline phosphatase activity indicates biliary-related liver damage, inflammatory intestinal diseases and/or increased bone formation (Reichling and Kaplan, 1988). Liver oxidative stress which result in hepatocyte damage and liver inflammation can occur due to fructose-induced excessive liver lipid accretion (Choi et al., 2017). My findings demonstrate that high-fructose diet caused fatty liver disease in the rats, without elevating the plasma activities of alkaline phosphatase or aspartate transaminase. Thus, I speculate that high-fructose diet did not cause hepatocyte damage or biliary-related liver damage in the rats. My findings could be ascribed to the fact that the diet-induced fatty liver disease did not lead to the development of NASH which is associated with hepatocellular damage.

Scientific authentication of medicinal plant extracts reduces the risk of hepatotoxicity that can result from the use of ethnomedicines. The crude hydroethanolic *M. oleifera* seed extract did not affect the plasma activities of alkaline phosphatase and aspartate transaminase in the rats,

suggesting that it could be employed for medicinal purposes without causing liver damage. On the contrary, fenofibrate increased plasma activity of alkaline phosphatase in male rats (Table 4.4). Thus, one could speculate that fenofibrate usage causes biliary-related liver damage in the rats. However, caution must be exercised when interpreting my result since alkaline phosphatase is none distinct biomarker for biliary-related liver damage.

A key finding of my study was that fructose caused metabolic dysfunction in sex-dependent manner as documented by other researchers (Galipeau et al., 2015; Pektaş et al., 2015; Sharma et al., 2015). Previous studies have found that female organisms are less prone to fructose-induced metabolic derangements due to oestrogen protective properties (Couchepin et al., 2008; Galipeau et al., 2015). Similarly, the crude hydroethanolic *M. oleifera* seed extract and fenofibrate exerted their activities in a sex-dependent manner. Nonetheless, there are several factors including epigenetics, in utero environment and the possibility of hereditary factors that can impact the response to nutritional stimuli in different sex organisms. Thus, at the moment I can not to offer comprehensive explanations for the sex-dependent findings of my study.

The next chapter outlines the conclusions and limitations of the study, as well as the recommendations.

CHAPTER 6: CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

Studies previously done on *M. oleifera* extracts have mostly focussed on the curative potential of the extracts. My study investigated the prophylactic potential of *M. oleifera* seed extract against fructose-induced metabolic derangements in growing rats, mimicking children fed obesogenic diets.

The fructose-diet and the crude hydroethanolic *M. oleifera* seed extract had no adverse effects on body mass gain of the rats. In male rats fed a fructose-diet only the administration of fenofibrate (positive control) reduced the terminal body masses. My study reports that fructose caused hypertriglyceridemia and fatty liver disease in male and female rats, visceral obesity, hyperinsulinemia and insulin resistance in female rats only. The crude hydroethanolic *M. oleifera* seed extract (high dose*)* prevented the development of fructose-induced fatty liver disease in male rats but not in female rats. Similarly, the crude hydroethanolic *M. oleifera* seed extract protected male and female rats against hypertriglyceridemia and in female rats only against insulin resistance and hyperinsulinemia. However, the present study demonstrated that in female rats the crude hydroethanolic *M. oleifera* seed extract did not prevent fructose-induced visceral adiposity.

In conclusion, I report that high-fructose diet feeding for 12-weeks causes metabolic derangements in a sex-dependent manner. Similarly, the crude hydroethanolic *M. oleifera* seed extract prevented certain components of the fructose-induced metabolic dysfunctions in a dose-dependent and sex-dependent way. Therefore, the crude hydroethanolic *M. oleifera* seed extract may potentially be used as a preventative intervention against fructose-induced metabolic dysfunctions in children.

The current study only determined total cholesterol, insulin and adiponectin concentrations. The concentrations of HDL, LDL, VLDL and leptin were not determined due to technical problems. While the presence of phytochemicals was determined, their quantification was not done. The renal health of the animals was also not assessed. The above-mentioned analyses which were not looked at in this study are recommended for future studies.

The present study outcomes have various implications on the planning of research investigating the potential prophylactic activities of both ethnomedicinal plants and synthetic pharmacological agents. Research interrogating these should therefore, consider the variation

in response to nutritional stimuli in males and females. Therefore, I advocate that future research should be based on both sexes to account for variances in sex-dependent responses.

CHAPTER 7: REFERENCES

Adamo, E.D., Guardamagna, O., Chiarelli, F., Bartuli, A., Liccardo, D., Ferrari, F., Nobili, V., 2015. Atherogenic Dyslipidemia and Cardiovascular Risk Factors in Obese Children. Int. J. Endrocrinology 2015, 1–9.

Agrawal, R., Gomez-Pinilla, F., 2012. "Metabolic syndrome" in the brain: Deficiency in omega-3 fatty acid exacerbates dysfunctions in insulin receptor signalling and cognition. J. Physiol. 590, 2485–2499.

Ahmed, H., Qader, S., Hussen, B., 2014. Prevalence of Metabolic Syndrome among Health Care Providers. Merit Res. J. Med. Med. Sci. 2, 238–244.

Ahmed, H.H., Metwally, F.M., Rashad, H., Zaazaa, A.M., Ezzat, S.M., Salama, M.M., 2014. Moringa oleifera offers a Multi-Mechanistic Approach for Management of Obesity in rats. Int. J. Pharm. Sci. Rev. Res. 29, 98–106.

Ajibade, T.O., Arowolo, R., Olayemi, F.O., 2013. Phytochemical screening and toxicity studies on the methanol extract of the seeds of moringa oleifera. J Complement Integr Med 10, 1–6.

Ajibola, M., Eunice, O., Stephanie, I.N., 2014. Effects of Aqueous Extract of Moringa oleifera Seeds on Alloxan Induced Hyperglycemia. Basic Sci. Med. 3, 37–42.

Al-Isa, A., 2013. Prevalence of metabolic syndrome (MS) among male kuwaiti adolescents aged 10-19 years. J. Diabetes 5, 123.

Al-Malki, A.L., El Rabey, H.A., 2015. The antidiabetic effect of low doses of moringa oleifera lam. Seeds on streptozotocin induced diabetes and diabetic nephropathy in male rats. Biomed Res. Int. 2015, 1–13.

Alhakmani, F., Kumar, S., Khan, S.A., 2013. Estimation of total phenolic content, in–vitro antioxidant and anti–inflammatory activity of flowers of Moringa oleifera. Asian Pac. J. Trop. Biomed. 3, 623–627.

Anderson, E.L., Howe, L.D., Jones, H.E., Higgins, J.P.T., Lawlor, D.A., Fraser, A., 2015. The prevalence of non-alcoholic fatty liver disease in children and adolescents: A systematic review and meta-analysis. PLoS One 10.

Aronne, L.J., Segal, K.R., 2002. Adiposity and fat distribution outcome measures: assessment and clinical implications. Obes. Res. 10 Suppl 1, 14S-21S.

Bais, S., Singh, G.S., Sharma, R., 2014. Antiobesity and Hypolipidemic Activity of Moringa oleifera Leaves against High Fat Diet-Induced Obesity in Rats. Adv. Biol. 2014, 1–9.

Barnes, P.M., Bloom, B., Nahin, R.L., 2007. Complementary and Alternative Medicine Use Among Adults and Children: United States, 2007.

Bays, H.E., Toth, P.P., Kris-Etherton, P.M., Abate, N., Aronne, L.J., Brown, W.V., Gonzalez-Campoy, J.M., Jones, S.R., Kumar, R., La Forge, R., Samuel, V.T., 2013. Obesity, adiposity, and dyslipidemia: A consensus statement from the National Lipid Association. J. Clin. Lipidol. 7, 304–383.

Beatrice Mpora, O., Adong Barbara, E.O., Francis, O., 2014. Glucose Addiction and Glycemic Control in Type 2 Diabetes Mellitus: A Case Report. Endocrinol. Metab. Syndr. 03, 3–4.

Bellostas, N., Christian Sørensen, J., Nikiema, A., Sørensen, H., Pasternak, D., Kumar, S.,

Lim, T.K., Mbikay, M., Atawodi, S.E., Atawodi, J.C., Idakwo, G.A., Pfundstein, B., Bennett, R.N., Mellon, F.A., Foidl, N., Pratt, J.H., Dupont, M.S., Perkins, L., Kroon, P.A., Ecker, K.L.B., Olson, M.E., Fahey, J.W., Pandey, A., Pradheep, K., Gupta, R., Nayar, E.R., Bhandari, D.C., 2003. Biochemistry and 2 Veterinary Public Health & Preventive Medicine Departments, Ahmadu Bello University, Zaria, Nigeria; and 3 Institute for Toxicology & Cancer Risk Factors, German Cancer Research Centre, Heidelberg, Germany. Front. Pharmacol. 3, 1071–1082.

Benzie, I.., Wachtel-Galor, S., 2011. Herbal Medicine: Biomolecular and Clinical Aspects, Second Edition - Google Books. In: Herbal Medicine: Biomolecular and Clinical Aspects, Second Edition.

Beysen, C., Ruddy, M., Stoch, A., Mixson, L., Rosko, K., Riiff, T., Turner, S.M., Hellerstein, M.K., Murphy, E.J., 2018. Dose-dependent quantitative effects of acute fructose administration on hepatic de novo lipogenesis in healthy humans. Am. J. Physiol. Metab. 315, E126–E132.

Bi, S., Moran, T.H., 2016. Obesity in the Otsuka Long Evans Tokushima Fatty Rat: Mechanisms and Discoveries. Front. Nutr. 3, 1–5.

Boden, G., Shulman, G.I., 2002. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. Eur. J. Clin. Invest. 32, 14–23.

Borza, C., Muntean, D., Dehelean, C., Savoiu, G., Serban, C., Simu, G., Andoni, M., Butur, M., Dragon, S., 2013. Oxidative Stress and Lipid Peroxidation - ALipid Metabolism Dysfunction.

Brands, M.W., Manhiani, M.M., 2012. Sodium-retaining effect of insulin in diabetes. Am. J. Physiol. Integr. Comp. Physiol. 303, R1101–R1109.

Bray, G.A., 2013. Why do we need drugs to treat the patient with obesity? Obesity.

Bray, G.A., Popkin, B.M., 2013. Calorie-sweetened beverages and fructose: What have we learned 10 years later. Pediatr. Obes. 8, 242–248.

Bridgewater, L.C., Zhang, C., Wu, Y., Hu, W., Zhang, Q., Wang, J., Li, S., Zhao, L., 2017. Gender-based differences in host behavior and gut microbiota composition in response to high fat diet and stress in a mouse model. Sci. Rep. 7, 1–12.

Buzzetti, E., Pinzani, M., Tsochatzis, E.A., 2016. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism 65, 1038–1048.

Byrne, C.D., 2013. Ectopic fat, insulin resistance and non-alcoholic fatty liver disease. Proc. Nutr. Soc. 72, 412–419.

Caldwell, J., 1989. The Biochemical Pharmacology of Fenofibrate. Cardiology 76, 33–44.

Cameron, A., Roubos, I., Ewen, M., Mantel-Teeuwisse, A.K., Leufkens, H.G.M., Laing, R.O., 2011. Differences in the availability of medicines for chronic and acute conditions in the public and private sectors of developing countries. Bull. World Health Organ. 89, 412–421.

Carrera-Bastos, P., Fontes-villalba, M., O'Keefe, J.H., Lindenberg, S., Cordain, L., 2011. The western diet and lifestyle and diseases of civilization. Res. Reports Clin. Cardiol. 2, 15–35.

Chalasani, N., Younossi, Z., Lavine, J.E., Diehl, A.M., Brunt, E.M., Cusi, K., Charlton, M., Sanyal, A.J., 2012. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 142, 1592–1609.

Chattopadhyay, S., Maiti, S., Maji, G., Deb, B., Pan, B., Ghosh, D., 2011. Protective role of Moringa oleifera (Sajina) seed on arsenic-induced hepatocellular degeneration in female albino rats. Biol. Trace Elem. Res. 142, 200–212.

Cheenpracha, S., Park, E.J., Yoshida, W.Y., Barit, C., Wall, M., Pezzuto, J.M., Chang, L.C., Chee, L., Chang, L.C., 2010. Potential anti-inflammatory phenolic glycosides from the medicinal plant Moringa oleifera fruits. Bioorganic Med. Chem. 18, 6598–6602.

Chen, C.Y.O., Crott, J., Liu, Z., Smith, D.E., 2010. Fructose and saturated fats predispose hyperinsulinemia in lean male rat offspring. Eur. J. Nutr. 49, 337–343.

Chen, L., Chen, R., Wang, H., Liang, F., 2015. Mechanisms Linking Inflammation to Insulin Resistance. Int. J. Endocrinol. 2015, 9 pages.

Chinedu, A.A., Alani, S.O., Olaide, A.O., 2015. Effect of the ethanolic leaf extract of Moringa oleifera on insulin resistance in streptozotocin induced diabetic rats. J. Plant Sci. 2, 5–12.

Choi, Y., Abdelmegeed, M.A., Song, B.J., 2017. Diet high in fructose promotes liver steatosis and hepatocyte apoptosis in C57BL/6J female mice: Role of disturbed lipid homeostasis and increased oxidative stress. Food Chem. Toxicol. 103, 111–121.

CIA, 2016. Obesity - Adult Prevalence Rate: The World Factbook [WWW Document]. URL https://www.cia.gov/library/publications/resources/the-world-factbook/docs/guidetowfbook.html (accessed 11.29.18).

Climate-Data.org, 2017. Pretoria Climate: Average Temperature, Weather by Month, Pretoria Weather Averages. Climate-Data.org [WWW Document]. URL https://en.climate-data.org/africa/south-africa/gauteng/pretoria-154/ (accessed 11.29.18).

Couchepin, C., Kim-Anne, L. Bortolotti, M., Encarnacao, J.A. Da, Oboni, J.B., Tran, C., Schneiter, P., Tappy, L., 2008. Markedly blunted metabolic effects of fructose in healthy young female subjects compared with male subjects. Diabetes Care 31, 1254–1256.

Cristina, D., Masquio, L., Ganen, A.D.P., Munhoz, R., Sanches, P.D.L., Corgosinho, F.C., Caranti, D., Tock, L., 2015. Cut-off values of waist circumference to predict metabolic syndrome in obese adolescents. Nutr Hosp. 31, 1540–1550.

Dangarembizi, R., Erlwanger, K., Chivandi, E., 2014. Effects of Ficu thonningii Extracts on the Gastrointestinal Tract and Clinical Biochemistry of Suckling Rats. Afr J Tradit Complement Altern Med 11, 285–291.

Das, B.R., Narasimha Rao, P.L., 1958. Antibiotic principle from Moringa pterygosperma. IX. Inhibition of transaminase by isothiocyanates. Indian J. Med. Res. 46, 75–77.

Das, N., Sikder, K., Ghosh, S., Fromenty, B., Dey, S., 2012. Moringa oleifera lam. leaf extract prevents early liver injury and restores antioxidant status in mice fed with high-fat diet. Indian J. Exp. Biol. 50, 404–412.

de Freitas Junior, L.M., de Almeida, E.B., 2017. Medicinal plants for the treatment of

obesity: ethnopharmacological approach and chemical and biological studies. Am. J. Transl. Res. 9, 2050–2064.

de Mello, M.T., de Piano, A., Carnier, J., Sanches, P. de L., Corrêa, F.A., Tock, L., Ernandes, R.M.Y., Tufik, S., Dâmaso, A.R., 2011. Long-Term Effects of Aerobic Plus Resistance Training on the Metabolic Syndrome and Adiponectinemia in Obese Adolescents. J. Clin. Hypertens. 13, 343–350.

Deepa, B., Suchetha, K., Rao, S., 2013. Antidepressant activity of nardostachys jatamansi in electron beam irradiated mice. Int. J. Res. Ayurveda Pharm. 4, 101–103.

Dekker, M.J., Su, Q., Baker, C., Rutledge, A.C., Adeli, K., 2010. Fructose: a highly lipogenic nutrient implicated in insulin resistance, hepatic steatosis, and the metabolic syndrome. AJP Endocrinol. Metab. 299, E685–E694.

Del Campo, J.A., Gallego, P., Grande, L., 2018. Role of inflammatory response in liver diseases: Therapeutic strategies. World J. Hepatol. 10, 1–7.

Delelegn, A., Sahile, S., Husen, A., 2018. Water purification and antibacterial efficacy of Moringa oleifera Lam. Agric. Food Secur. 7, 1–10.

Dheer, R., Bhatnagar, P., 2010. A study of the antidiabetic activity of Barleria prionitis Linn. Indian J. Pharmacol. 42, 69.

Dunning, K.R., Anastasi, M.R., Zhang, V.J., Russell, D.L., Robker, R.L., 2014. Regulation of fatty acid oxidation in mouse cumulus-oocyte complexes during maturation and modulation by PPAR agonists. PLoS One 9, 1–11.

Ejikeme, C.M., Ezeonu, C.S., Eboatu, A.N., 2014. Determination of Physical and Phytochemical Constituents of Some Tropical Timbers Indigenous To Niger Delta Area of Nigeria. Eur. Sci. J. 10, 1857–7881.

Elangovan, M., Dhanarajan, M.S., Elangovan, I., 2015. Determination of Bioactive Compounds from the Petroleum Ether Extract of Moringa oleifera and Phyllanthus emblica using GC-MS Analysis. World J. Pharm. Res. 4, 1284–1298.

Fabbrini, E., Mohammed, B.S., Korenblat, K.M., Magkos, F., McCrea, J., Patterson, B.W., Klein, S., 2010. Effect of fenofibrate and niacin on intrahepatic triglyceride content, very low-density lipoprotein kinetics, and insulin action in obese subjects with nonalcoholic fatty liver disease. J. Clin. Endocrinol. Metab. 95, 2727–2735.

Fadeyi, A., Raheem, R., Njan, A., Olatunji, L., Afolabi, S., Olorundare, O., 2015. Antibacterial Activity of the Crude Extracts of Moringa Oleifera Leaf Lam. Niger. J. Pharm. Sci. 14, 1–9.

Fahey, J., 2005. Moringa oleifera: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic Properties. Part 1. Trees life J. 1–15.

Faizi, S., Siddiqui, B.S., Saleem, R., Aftab, K., Shaheen, F., Gilani, A.U.H., 1998. Hypotensive constituents from the pods of Moringa oleifera. Planta Med. 64, 225–228.

Farnier, M., 2008. Update on the clinical utility of feno fi brate in mixed dyslipidemias : mechanisms of action and rational prescribing. Vasc. Health Risk Manag. 4, 991–1000.

Fawole, O.A., Ndhlala, A.R., Amoo, S.O., Finnie, J.F., Van Staden, J., 2009. Anti-inflammatory and phytochemical properties of twelve medicinal plants used for treating

gastro-intestinal ailments in South Africa. J. Ethnopharmacol. 123, 237–243.

Ferder, L., Ferder, M.D., Inserra, F., 2010. The role of high-fructose corn syrup in metabolic syndrome and hypertension. Curr. Hypertens. Rep.

Ferreira, A.V.M., Parreira, G.G., Porto, L.C.J., Mario, É.G., Delpuerto, H.L., Martins, A.S., Botion, L.M., 2008. Fenofibrate prevents orotic acid-Induced hepatic steatosis in rats. Life Sci. 82, 876–883.

Friend, A., Craig, L., Turner, S., 2013. The Prevalence of Metabolic Syndrome in Children: A Systematic Review of the Literature. Metab. Syndr. Relat. Disord. 11, 71–80.

Galipeau, D., Verma, S., McNeill, J.H., 2015. Female rats are protected against fructose-induced changes in metabolism and blood pressure. Am. J. Physiol. Circ. Physiol. 283, H2478–H2484.

Gao, Y., Shen, W., Lu, B., Zhang, Q., Hu, Y., Chen, Y., 2014. Upregulation of hepatic VLDLR via PPARα is required for the triglyceride-lowering effect of fenofibrate. J. Lipid Res. 55, 1622–1633.

Garnett, S.P., Baur, L.A., Srinivasan, S., Lee, J.W., Cowell, C.T., 2007. Body mass index and waist circumference in midchildhood and adverse cardiovascular disease risk clustering in adolescence. Am. J. Cinical Nutr. 86, 549–55.

GBD, 2017. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. N. Engl. J. Med. 377, 13–27.

Ghasi, S., Nwobodo, E., Ofili, J.O., 2000. Hypocholesterolemic effects of crude extract of leaf of Moringa oleifera Lam in high-fat diet fed wistar rats. J. Pharmacol. 69, 21.

Ghezzi, A., Cambri, L., Botezelli, J., Ribeiro, C., Dalia, R., de Mello, M.A., 2012. Metabolic syndrome markers in wistar rats of different ages. Diabetol. Metab. Syndr. 4, 16.

Gluba, A., Mikhailidis, D.P., Lip, G.Y.H., Hannam, S., Rysz, J., Banach, M., 2013. Metabolic syndrome and renal disease. Int. J. Cardiol. 164, 141–150.

González-Castejón, M., Rodriguez-Casado, A., 2011. Dietary phytochemicals and their potential effects on obesity: A review. Pharmacol. Res.

Govardhan Singh, R.S., Negi, P.S., Radha, C., 2013. Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of Moringa oleifera seed flour. J. Funct. Foods 5, 1883–1891.

Grave, R.., Calugi, S., Centis, E., Marzocchi, R., El Ghoch, M., Marchesini, G., 2010. Lifestyle modification in the management of the metabolic syndrome: achievements and challenges. Diabetes, Metab. Syndr. Obes. Targets Ther. 3, 373.

Grundy, S.M., 1999. Hypertriglyceridemia, insulin resistance, and the metabolic syndrome. Am. J. Cardiol. 83, 25–29.

Guevara, A.P., Vargas, C., Sakurai, H., Fujiwara, Y., Hashimoto, K., Maoka, T., Kozuka, M., Ito, Y., Tokuda, H., Nishino, H., 1999. An antitumor promoter from Moringa oleifera Lam. Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 440, 181–188.

Guo, L., Gao, Z., Zhang, L., Guo, F., Chen, Y., Li, Y., Huang, C., 2016. Saponin-enriched sea cucumber extracts exhibit an antiobesity effect through inhibition of pancreatic lipase activity and upregulation of LXR-β signaling. Pharm. Biol. 54, 1312–1325.

Guo, S., 2014. Decoding insulin resistance and metabolic syndrome for promising therapeutic intervention. J. Endocrinol. 220, E1–E3.

Gupta, R., Mathur, M., Bajaj, V.K., Katariya, P., Yadav, S., Kamal, R., Gupta, R.S., 2012. Evaluation of antidiabetic and antioxidant activity of Moringa oleifera in experimental diabetes 4, 164–171.

Gustafson, B., Gogg, S., Hedjazifar, S., Jenndahl, L., Hammarstedt, A., Smith, U., 2009. Inflammation and impaired adipogenesis in hypertrophic obesity in man. Am. J. Physiol. Endocrinol. Metab. 297, E999–E1003.

Halaby, M.S., El-Din, M.M., Emara, N.E., 2015. Influence of Moringa oleifera on Non-Alcoholic Fatty Liver in Adult Albino Rats. Middle East J. Appl. Sci. 5, 902–912.

Hamza, A.A., 2010. Ameliorative effects of Moringa oleifera Lam seed extract on liver fibrosis in rats. Food Chem. Toxicol. 48, 345–355.

Hassan, H.A., El-Gharib, N.E., 2015. Obesity and Clinical Riskiness Relationship: Therapeutic Management by Dietary Antioxidant Supplementation—a Review. Appl. Biochem. Biotechnol.

Helvaci, M.R., Ozcan, A., 2015. White Coat Hypertension is a Pioneer Sign of Metabolic Syndrome. J. Metab. Syndr. 04, 8–11.

Hu, F.B., Malik, V.S., 2010. Sugar-sweetened beverages and risk of obesity and type 2 diabetes: Epidemiologic evidence. Physiol. Behav. 100, 47–54.

Hukkeri, V., Nagathan, C., Karadi, R., Patil, B., 2006. Antipyretic and wound healing activities of moringa oleifera lam. in rats. Indian J. Pharm. Sci. 68, 124.

IDF, 2006. The IDF consensus worldwide definition of the metabolic syndrome, International Diabetes Federation.

Idris, M.A., Mohammed, S.J., Ademola, M.H., Parveen, J., 2016. Moringa oleifera seed extract: A review on its environmental applications. Int. J. Appl. Environ. Sci 11, 1469–1486.

Jain, P.G., Patil, S.D., Haswani, N.G., Girase, M. V, Surana, S.J., 2010. Atividade hipolipidemica de Moringa oleifera Lam., Moringaceae, na hiperlipidemia induzida por dieta rica em gordura em ratos albinos. Brazilian J. Pharmacogn. 20, 969–973.

Jaiswal, D., Kumar Rai, P., Kumar, A., Mehta, S., Watal, G., Rai, P.K., Kumar, A., Mehta, S., Watal, G., 2009. Effect of Moringa oleifera Lam. leaves aqueous extract therapy on hyperglycemic rats. J. Ethnopharmacol. 123, 392–396.

Jaiswal, D., Rai, P.K., Mehta, S., Chatterji, S., Shukla, S., Rai, D.K., Sharma, G., Sharma, B., Khair, S., Watal, G., 2013. Role of Moringa oleifera in regulation of diabetes-induced oxidative stress. Asian Pac. J. Trop. Med. 6, 426–432.

Jatkar, A., Kurland, I.J., Judex, S., 2017. Diets High in Fat or Fructose Differentially Modulate Bone Health and Lipid Metabolism. Calcif. Tissue Int. 100, 20–28.

Jo, S.P., Kim, J.K., Lim, Y.H., 2014. Antihyperlipidemic effects of stilbenoids isolated from Morus alba in rats fed a high-cholesterol diet. Food Chem. Toxicol. 65, 213–218.

Kadowaki, T., Toshimasa, T., Naoto, N., Kazuo, K., Kohjiro, K., Kazuyuki, K., 2006. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic

syndrome. Journal of Clinical Investigation. J. Clin. Invest. 116, 1784–1792.

Kamerman, P.R., Modisa, B.M.E., Mphahlele, N.R., 2004. Atorvastatin , a potent HMG-CoA reductase inhibitor , is not antipyretic in rats. J. Therm. Biol. 29, 431–435.

Katsumata, Y., Todoriki, H., Higashiuesato, Y., Yasura, S., Willcox, D.C., Ohya, Y., Willcox, B.J., Dodge, H.H., 2012. Metabolic syndrome and cognitive decline among the oldest old in Okinawa: In search of a mechanism. The KOCOA project. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 67 A, 126–134.

Kaur, J., 2014. A comprehensive review on metabolic syndrome. Cardiol. Res. Pract.

Kaur, J., Young, B.E., Fadel, P.J., 2017. Sympathetic overactivity in chronic kidney disease: Consequences and mechanisms. Int. J. Mol. Sci. 18.

Kazemipoor, M., Wan, C., Wan, J., Radzi, M., Cordell, G.A., 2012. Potential of Traditional Medicinal Plants for Treating Obesity : A Review. In: 2012 International Conference on Nutrition and Food Sciences, IPCBEE. IACSIT Press, Singapore, p. 5 pages.

Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Torbenson, M.S., Unalp-Arida, A., Yeh, M., McCullough, A.J., Sanyal, A.J., 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41, 1313–1321.

Klop, B., Elte, J.W.F., Cabezas, M.C., 2013. Dyslipidemia in Obesity: Mechanisms and Potential Targets. Nutrients.

Kneeman, J.M., Misdraji, J., Corey, K.E., 2012. Secondary causes of nonalcoholic fatty liver disease. Therap. Adv. Gastroenterol. 5, 199–207.

Kooltheat, N., Pankla Sranujit, R., Chumark, P., Potup, P., Laytragoon-Lewin, N., Usuwanthim, K., 2014. An ethyl acetate fraction of Moringa oleifera Lam. inhibits human macrophage cytokine production induced by cigarette smoke. Nutrients 6, 697–710.

Kosuru, R., Singh, S., 2017. Pterostilbene ameliorates insulin sensitivity, glycemic control and oxidative stress in fructose-fed diabetic rats. Life Sci. 182, 112–121.

Kraja, A.T., Province, M.A., Straka, R.J., Ordovas, J.M., Borecki, I.B., Arnett, D.K., 2010. Fenofibrate and metabolic syndrome. Endocr. Metab. Immune Disord. Drug Targets 10, 138–148.

Krishnaiah Bono, D.R.S.A., 2007. Phytochemical antioxidants for health and medicine - A move towards nature. Biotechnol. Mol. Biol. rfeview Vol. 1 pp.097-104, Sept. 2007 1, 97–104.

Kumbhare, M.R., Guleha, V., Sivakumar, T., 2012. Estimation of total phenolic content, cytotoxicity and in-vitro antioxidant activity of stem bark of Moringa oleifera. Asian Pacific J. Trop. Dis. 2, 144–150.

Landrault, N., Poucheret, P., Ravel, P., Gasc, F., Cros, G., Teissedre, P.L., 2001. Antioxidant capacities and phenolics levels of French wines from different varieties and vintages. J. Agric. Food Chem. 49, 3341–3348.

Larosa, J.C., Pedersen, T.R., Somaratne, R., Wasserman, S.M., 2013. Safety and effect of very low levels of low-density lipoprotein cholesterol on cardiovascular events. Am. J.

Cardiol.

Last, A.R., Ference, J.D., Falleroni, J., 2011. Pharmacologic treatment of hyperlipidemia. Am. Fam. Physician 84, 551–558.

Leggio, M., Lombardi, M., Caldarone, E., Severi, P., D'Emidio, S., Armeni, M., Bravi, V., Bendini, M.G., Mazza, A., 2017. The relationship between obesity and hypertension: an updated comprehensive overview on vicious twins. Hypertens. Res. 40, 947–963.

Lehnen, A.M., Rodrigues, B., Irigoyen, M.C., De Angelis, K., Schaan, B.D.A., 2013. Cardiovascular changes in animal models of metabolic syndrome. J. Diabetes Res. 2013, 761314.

Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., Bertoli, S., 2016. Moringa oleifera Seeds and Oil : Characteristics and Uses for Human Health. Int. J. Mol. Sci. 17, 1–14.

Leong, X.F., Ng, C.Y., Jaarin, K., 2015. Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis. Biomed Res. Int. 2015.

Li, N., Fu, J., Koonen, D.P., Kuivenhoven, J.A., Snieder, H., Hofker, M.H., 2014. Are hypertriglyceridemia and low HDL causal factors in the development of insulin resistance? Atherosclerosis 233, 130–138.

Lim, J.S., Mietus-Snyder, M., Valente, A., Schwarz, J.M., Lustig, R.H., 2010. The role of fructose in the pathogenesis of NAFLD and the metabolic syndrome. Nat. Rev. Gastroenterol. Hepatol. 7, 251–264.

Lin, M., Zhang, J., Chen, X., 2018. Bioactive flavonoids in Moringa oleifera and their health-promoting properties. J. Funct. Foods 47, 469–479.

Ling, H., Luoma, J.T., Hilleman, D., 2013. A Review of Currently Available Fenofibrate and Fenofibric Acid Formulations. Rev. Cardiol Res @BULLET 4, 47–55.

Liu, R.H., 2013. Health-Promoting Components of Fruits and Vegetables in the Diet. Adv. Nutr. An Int. Rev. J. 4, 384S-392S.

Liu, S. nan, Liu, Q., Li, L. yi, Huan, Y., Sun, S. juan, Shen, Z. fang, 2011. Long-term fenofibrate treatment impaired glucose-stimulated insulin secretion and up-regulated pancreatic NF-kappa B and iNOS expression in monosodium glutamate-induced obese rats: Is that a latent disadvantage? J. Transl. Med. 9, 1–11.

Locatelli, F., Pozzoni, P., Del Vecchio, L., 2006. Renal Manifestations in the Metabolic Syndrome: Table 1. J. Am. Soc. Nephrol. 17, S81–S85.

Lutz, T.A., Woods, S.C., 2012. Overview of animal models of obesity. Curr. Protoc. Pharmacol. 5, 1–22.

Ma, K.L., Ruan, X.Z., Powis, S.H., Chen, Y., Moorhead, J.F., Varghese, Z., 2008. Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. Hepatology 48, 770–781.

Mackenbach, J.P., Roskam, A.R., Schaap, M.M., Menvielle, G., Leinsalu, M., Kunst, A.E., Sc, M., Schaap, M.M., Sc, M., 2008. Socioeconomic Inequalities in Health in 22 European Countries. N. Engl. J. Med. 359, 1290–1291.

Mahajan, S.G., Banerjee, A., Chauhan, B.F., Padh, H., Nivsarkar, M., Mehta, A.A., 2009.

Inhibitory effect of n-butanol fraction of Moringa oleifera Lam. seeds on ovalbumin-induced airway inflammation in a guinea pig model of asthma. Int. J. Toxicol. 28, 519–527.

Mahajan, S.G., Mali, R.G., Mehta, A.A., 2007. Protective effect of ethanolic extract of seeds of Moringa oleifera Lam. against inflammation associated with development of arthritis in rats. J. Immunotoxicol. 4, 39–47.

Mahajan, S.G., Mehta, A.A., 2010. Immunosuppressive activity of ethanolic extract of seeds of Moringa oleifera Lam. in experimental immune inflammation. J. Ethnopharmacol. 130, 183–186.

Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., Faria, A., Calhau, C., 2015. High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 5, 11–21.

Marrelli, M., Conforti, F., Araniti, F., Statti, G.A., 2016. Effects of saponins on lipid metabolism: A review of potential health benefits in the treatment of obesity. Molecules 21.

Mather, K.J., Steinberg, H.O., Baron, A.D., 2013. Insulin resistance in the vasculature. J. Clin. Invest. 123, 1003–1004.

Matough, F.A., Budin, S.B., Hamid, Z.A., Alwahaibi, N., Mohamed, J., 2012. The Role of Oxidative Stress and Antioxidants in Diabetic Complications. SQU Med J 12, 5–18.

Matson, K.L., Fallon, R.M., 2012. Treatment of Obesity in Children and Adolescents. J Pediatr Pharmacol Ther 17, 45–57.

Matsuda, M., Shimomura, I., 2013. Increased oxidative stress in obesity : Implications for metabolic syndrome , diabetes , hypertension , dyslipidemia , atherosclerosis , and cancer. Obes. Res. Clin. Pract. 7, e330–e341.

Matthews, D., Hosker, J., Rudenski, A., Naylor, B., Treacher, D., Turner, R., 1985. Homeostasis model assessment: Insulin resistance and beta- cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28, 412–419.

Mauvais-Jarvis, F., 2015. Sex differences in metabolic homeostasis, diabetes, and obesity. Biol. Sex Differ. 6, 1–9.

Mbikay, M., 2012. Therapeutic potential of Moringa oleifera leaves in chronic hyperglycemia and dyslipidemia: A review. Front. Pharmacol. 3, 1–12.

Mehta, L.K., Balaraman, R., Amin, A.H., Bafna, P.A., Gulati, O.D., Komal, L., Balaraman, R., Amin, A.H., Bafna, P.A., Gulati, O.D., 2003. Effect of fruits of Moringa oleifera on the lipid profile of normal and hypercholesterolaemic rabbits. J. Ethnopharmacol. 86, 191–195.

Ménard, S.L., Croteau, E., Sarrhini, O., Gélinas, R., Brassard, P., Ouellet, R., Bentourkia, M., van Lier, J.E., Rosiers, C. Des, Lecomte, R., Carpentier, A.C., 2010. Abnormal in vivo myocardial energy substrate uptake in diet-induced type 2 diabetic cardiomyopathy in rats. Am. J. Physiol. Metab. 298, E1049–E1057.

Mensah, J.K., Ikhajiagbe, B., Edema, N.E., Emokhor, J., 2012. Phytochemical , nutritional and antibacterial properties of dried leaf powder of Moringa oleifera (Lam) from Edo Central Province , Nigeria. J. Nat. Prod. Plant Resour. 2, 107–112.

Metwally, F.M., Rashad, H.M., Ahmed, H.H., Mahmoud, A.A., Abdol Raouf, E.R., Abdalla, A.M., 2017. Molecular mechanisms of the anti-obesity potential effect of Moringa oleifera in the experimental model. Asian Pac. J. Trop. Biomed. 7, 214–221.

Minaiyan, M., Asghari, G., Taheri, D., Saeidi, M., Nasr-Esfahani, S., 2014. Anti-inflammatory effect of Moringa oleifera Lam. seeds on acetic acid-induced acute colitis in rats. Avicenna J Phytomed 4, 127–136.

Mishra, G., Singh, P., Verma, R., Kumar, S., Srivastav, S., Jha, K.K., Khosa, R.L., 2011. Traditional uses, phytochemistry and pharmacological properties of Moringa oleifera plant: An overview. Der Pharm. Lett. 3, 141–164.

Mohamed, G.A., Ibrahim, S.R.M., Elkhayat, E.S., El Dine, R.S., 2014. Natural anti-obesity agents. Bull. Fac. Pharmacy, Cairo Univ. 52, 269–284.

Montezano, A.C., Touyz, R.M., 2012. Molecular Mechanisms of Hypertension-Reactive Oxygen Species and Antioxidants: A Basic Science Update for the Clinician. Can. J. Cardiol. 28, 288–295.

Monyeki, M.A., Awotidebe, A., Strydom, G.L., De Hans Ridder, J., Mamabolo, R.L., Han, C.G., 2015. The challenges of underweight and overweight in South African children: Are we winning or losing the battle? A systematic review. Int. J. Environ. Res. Public Health 12, 1156–1173.

Moschen, A.R., Molnar, C., Geiger, S., Graziadei, I., Ebenbichler, C.F., Weiss, H., Kaser, S., Kaser, A., Tilg, H., 2010. Anti-inflammatory effects of excessive weight loss: Potent suppression of adipose interleukin 6 and tumour necrosis factor a expression. Gut 59, 1259–1264.

Muangnoi, C., Chingsuwanrote, P., Praengamthanachoti, P., Svasti, S., Tuntipopipat, S., 2012. Moringa oleifera pod inhibits inflammatory mediator production by lipopolysaccharide-stimulated RAW 264.7 murine macrophage cell lines. Inflammation 35, 445–455.

Mukhopadhyay, S.K., 2012. Study of Lipid Profile in Obese Individuals and the Effect of Cholesterol Lowering Agents on Them. Al Ameen J Med Sci 5, 147–151.

Nahar, S., Faisal, F., Iqbal, J., Rahman, M., Yusuf, M., 2016. Antiobesity activity of Moringa oleifera leaves against high fat diet-induced obesity in rats. Int. J. Basic Clin. Pharmacol. 5, 1263–1268.

Navarro-Millán, I., Yang, S., DuVall, S.L., Chen, L., Baddley, J., Cannon, G.W., Delzell, E.S., Zhang, J., Safford, M.M., Patkar, N.M., Mikuls, T.R., Singh, J.A., Curtis, J.R., 2016. Association of hyperlipidaemia, inflammation and serological status and coronary heart disease among patients with rheumatoid arthritis: Data from the National Veterans Health Administration. Ann. Rheum. Dis. 75, 341–347.

Nazaruk, J., Borzym-Kluczyk, M., 2015. The role of triterpenes in the management of diabetes mellitus and its complications. Phytochem. Rev. 14, 675–690.

Ndong, M., Uehara, M., Katsumata, S., Suzuki, K., 2007. Effects of Oral Administration of Moringa oleifera Lam on Glucose Tolerance in Goto-Kakizaki and Wistar Rats. J. Clin. Biochem. Nutr. 40, 229–233.

Nettleton, J.A., Jebb, S., Riserus, U., Koletzko, B., Fleming, J., 2014. Role of Dietary Fats in the Prevention and Treatment of the Metabolic Syndrome. Ann. Nutr. Metab. 64, 167–

178.

Niswender, K.D., Daws, L.C., Avison, M.J., Galli, A., 2011. Insulin Regulation of Monoamine Signaling: Pathway to Obesity. Neuropsychopharmacology 36, 359–360.

Nunes-Souza, V., César-Gomes, C.J., Da Fonseca, L.J.S., Guedes, G.D.S., Smaniotto, S., Rabelo, L.A., 2016. Aging Increases Susceptibility to High Fat Diet-Induced Metabolic Syndrome in C57BL/6 Mice: Improvement in Glycemic and Lipid Profile after Antioxidant Therapy. Oxid. Med. Cell. Longev. 2016, 1–17.

Olson, M.E., Sankaran, R.P., Fahey, J.W., Grusak, M.A., Odee, D., Nouman, W., 2016. Leaf protein and mineral concentrations across the "Miracle tree" genus moringa. PLoS One.

Ouchi, N., Walsh, K., 2007. Adiponectin as an anti-inflammatory factor. Clin. Chim. Acta 380, 24–30.

Oyedepo, T.A., Babarinde, S.O., Ajayeoba, T.A., 2013. Evaluation of Anti-hyperlipidemic Effect of Aqueous Leaves Extract of Moringa oleifera in Alloxan Induced Diabetic Rats. Int. J. Biochem. Res. Rev. 3, 162–170.

Palhares, R.M., Drummond, M.G., Dos Santos Alves Figueiredo Brasil, B., Cosenza, G.P., Das Graças Lins Brandão, M., Oliveira, G., 2015. Medicinal plants recommended by the world health organization: DNA barcode identification associated with chemical analyses guarantees their quality. PLoS One 10, e0127866.

Paliwal, R., Sharma, V., . P., 2011. A Review on Horse Radish Tree (Moringa oleifera): A Multipurpose Tree with High Economic and Commercial Importance. Asian J. Biotechnol. 3, 317–328.

Pan, H., Guo, J., Su, Z., 2014. Advances in understanding the interrelations between leptin resistance and obesity. Physiol. Behav.

Panchal, S.K., Brown, L., 2011. Rodent Models for Metabolic Syndrome Research. J. Biomed. Biotechnol. 2011, 1–14.

Panchal, S.K., Poudyal, H., Iyer, A., Nazer, R., Alam, A., Diwan, V., Kauter, K., Sernia, C., Campbell, F., Ward, L., Gobe, G., Fenning, A., Brown, L., 2011. High-carbohydrate, High-fat Diet–induced Metabolic Syndrome and Cardiovascular Remodeling in Rats. J. Cardiovasc. Pharmacol. 57, 611–624.

Parasuraman, S., Balamurugan, S., Christapher, P., Petchi, R., Yeng, W., Sujithra, J., Vijaya, C., 2015. Evaluation of Antidiabetic and Antihyperlipidemic Effects of Hydroalcoholic Extract of Leaves of Ocimum tenuiflorum (Lamiaceae) and Prediction of Biological Activity of its Phytoconstituents. Pharmacognosy Res. 7, 156–165.

Pektaş, M.B., Sadi, G., Akar, F., 2015. Long-term dietary fructose causes gender-different metabolic and vascular dysfunction in rats: Modulatory effects of resveratrol. Cell. Physiol. Biochem. 37, 1407–1420.

Petäjä, E., Yki-Järvinen, H., 2016. Definitions of Normal Liver Fat and the Association of Insulin Sensitivity with Acquired and Genetic NAFLD—A Systematic Review. Int. J. Mol. Sci. 17, 633.

Petrovska, B., 2012. Historical review of medicinal plants′ usage. Pharmacogn. Rev. 6, 1.

Posmontier, B., 2011. The medicinal qualities of Moringa oleifera. Holist. Nurs. Pract. 25,

80–87.

Prasanna, K.K., Mandapaka, R.T., 2013. Effect of Moringa oleifera on Blood Glucose , LDL levels in Type II Diabetic Obese People. Innov. J. Med. Heal. Sci. 3, 23–25.

Raatz, S.K., Conrad, Z., Johnson, L.A.K., Picklo, M.J., Jahns, L., 2017. Relationship of the reported intakes of fat and fatty acids to body weight in US adults. Nutrients 9, 1–13.

Rana, J.S., Nieuwdorp, M., Jukema, J.W., Kastelein, J.J.P., 2007. Cardiovascular metabolic syndrome ? an interplay of, obesity, inflammation, diabetes and coronary heart disease. Diabetes, Obes. Metab. 9, 218–232.

Randriamboavonjy, J.I., Loirand, G., Vaillant, N., Lauzier, B., Derbré, S., Michalet, S., Pacaud, P., Tesse, A., 2016. Cardiac protective effects of moringa oleifera seeds in spontaneous hypertensive rats. Am. J. Hypertens. 29, 873–881.

Rani, J.G., Srilatha, D., 2015. Effects of Metabolic syndrome. Rsearch Rev. J. Med. Heal. Sci. 4, 1–8.

Razis, A.F.A., Ibrahim, M.D., Kntayya, S.B., Abdull Razis, A.F., Ibrahim, M.D., Kntayya, S.B., 2014. Health Benefits of Moringa oleifera. Asian Pacific J. Cancer Prev. 15, 8571–8576.

Reaven, G., 2004. The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. Endocrinol. Metab. Clin. North Am.

Regaieg, S., Charfi, N., Kamoun, S., Eulleuch, M., Marrakchi, R., jamoussi, K., Damak, J., Abid, M., 2015. Prevalence of metabolic syndrome and its components among overweight and obese secondary school adolescent in SFAX, Tunisia. Int. J. Clin. Nutr. 3, 1–6.

Reichling, J.J., Kaplan, M.M., 1988. Clinical use of serum enzymes in liver disease. Dig. Dis. Sci. 33, 1601–1614.

Roger, V.L., Go, A.S., Lloyd-jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., Simone, G. De, Ford, E.S., Fox, C.S., Fullerton, H.J., Gillespie, C., Greenlund, K.J., Hailpern, S.M., John, A., Ho, P.M., Howard, V.J., Kissela, B.M., Kittner, S.J., Lackland, D.T., Judith, H., Lisabeth, L.D., Makuc, D.M., Gregory, M., Marelli, A., Matchar, D.B., Mary, M., Meigs, J.B., Moy, C.S., Mussolino, M.E., Nichol, G., Paynter, N.P., Rosamond, W.D., Sorlie, P.D., Stafford, R.S., Turan, T.N., Turner, M.B., Wong, N.D., Wylie-rosett, J., 2011. Heart Disease and Stroke Statistics-2011 update. Circulation 123.

Roglans, N., 2002. Atorvastatin Treatment Induced Peroxisome Proliferator-Activated Receptor alpha Expression and Decreased Plasma Nonesterified Fatty Acids and Liver Triglyceride in Fructose-Fed Rats. J. Pharmacol. Exp. Ther. 302, 232–239.

Roh, C., Jung, U., 2012. Screening of crude plant extracts with anti-obesity activity. Int. J. Mol. Sci. 13, 1710–1719.

Rosini, T.C., Ramos da Silva, A.S., de Moraes, C., 2012. Diet-induced obesity: rodent model for the study of obesity-related disorders. Rev. da Assoc. Médica Bras. (English Ed. 58, 383–387.

Rossouw, H., Grant, C., Viljoen, M., 2012. Overweight and obesity in children and adolescents: The South African problem. S Afr J Sci 108, 1–7.

Rousset, X., Shamburek, R., Vaisman, B., Amar, M., Remaley, A.T., 2011. Lecithin cholesterol acyltransferase: An anti- or pro-atherogenic factor? Curr. Atheroscler. Rep. 13, 249–256.

Rutledge, A.C., Adeli, K., 2007. Fructose and the Metabolic Syndrome : Pathophysiology and Molecular Mechanisms. Nutrition 65, 13–23.

Saboya, P.P., Bodanese, L.C., Zimmermann, P.R., Gustavo, A. da S., Macagnan, F.E., Feoli, A.P., Oliveira, M. da S., 2016. Lifestyle Intervention on Metabolic Syndrome and its Impact on Quality of Life: A Randomized Controlled Trial. Arq. Bras. Cardiol. 60–69.

SADHS, 2016. South Africa Demographic and Health Survey. www.stassa.gov.za > publications.

Sakaguchi, K., Takeda, K., Maeda, M., Ogawa, W., Sato, T., Okada, S., Ohnishi, Y., Nakajima, H., Kashiwagi, A., 2016. Glucose area under the curve during oral glucose tolerance test as an index of glucose intolerance. Diabetol. Int. 7, 53–58.

Sartorius, B., Veerman, L.J., Manyema, M., Chola, L., Hofman, K., 2015. Determinants of obesity and associated population attributability, South Africa: Empirical evidence from a national panel survey, 2008-2012. PLoS One 10, 2008–2012.

Sashidhara, K. V, Rosaiah, J.N., Tyagi, E., Shukla, R., Raghubir, R., Rajendran, S.M., 2009. Rare dipeptide and urea derivatives from roots of Moringa oleifera as potential anti-inflammatory and antinociceptive agents. Eur. J. Med. Chem. 44, 432–436.

Schauer, P.R., Mingrone, G., Ikramuddin, S., Wolfe, B., 2016. Clinical outcomes of metabolic surgery: Efficacy of glycemic control, weight loss, and remission of diabetes. Diabetes Care 39, 902–911.

Scott, R., O'Brien, R., Fulcher, G., Pardy, C., D'Emden, M., Tse, D., Taskinen, M.R., Ehnholm, C., Keech, A., 2009. Effects of Fenofibrate Treatment on Cardiovascular Disease Risk in 9,795 Individuals With Type 2 Diabetes and Various Components of the Metabolic Syndrome The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. Diabetes Care 32, 493–498.

Serra, A., Romero, R., Lopez, D., Navarro, M., Esteve, A., Perez, N., Alastrue, A., Ariza, A., 2008. Renal injury in the extremely obese patients with normal renal function. Kidney Int. 73, 947–955.

Seyssel, K., Meugnier, E., Lê, K.-A., Durand, C., Disse, E., Blond, E., Pays, L., Nataf, S., Brozek, J., Vidal, H., Tappy, L., Laville, M., Le, K.-A., Durand, C., Disse, E., Blond, E., Pays, L., Nataf, S., Brozek, J., Vidal, H., Tappy, L., Laville, M., 2016. Fructose overfeeding in first-degree relatives of type 2 diabetic patients impacts energy metabolism and mitochondrial functions in skeletal muscle. Mol. Nutr. Food Res. 60, 2691–2699.

Shanker, K., Gupta, M.M., Srivastava, S.K., Bawankule, D.U., Pal, A., Khanuja, S.P.S., 2007. Food Chemistry Determination of bioactive nitrile glycoside (s) in drumstick (Moringa oleifera) by reverse phase HPLC. Food Chem. 105, 376–382.

Sharma, N., Li, L., Ecelbarger, C.M., 2015. Sex differences in renal and metabolic responses to a high-fructose diet in mice. Am. J. Physiol. Physiol. 308, F400–F410.

Siddhuraju, P., Becker, K. laus, 2003. Antioxidant Properties of Various Solvent Extracts of Total Phenolic Constituents from Three Different Agroclimatic Origins of Drumstick

Tree (Moringa oleifera Lam .) Leaves. J. Agric. Food Chem. 51, 2144–2155.

Singh, B.N., Singh, B.R., Singh, R.L., Prakash, D., Dhakarey, R., Upadhyay, G., Singh, H.B., 2009. Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of Moringa oleifera. Food Chem. Toxicol. 47, 1109–1116.

Singh, V.P., Bali, A., Singh, N., Jaggi, A.S., 2014. Advanced glycation end products and diabetic complications. Korean J. Physiol. Pharmacol. 18, 1–14.

Softic, S., Cohen, D.E., Kahn, C.R., 2016. Role of Dietary Fructose and Hepatic De Novo Lipogenesis in Fatty Liver Disease. Dig. Dis. Sci. 61, 1282–1293.

Song, Y., Zhang, X., Chen, W., Gao, L., 2017. Cholesterol Synthesis Increased in the MMI-Induced Subclinical Hypothyroidism Mice Model. Int. J. Endocrinol. 2017, 1–6.

Sponchiado, G., Adam, M.L., Silva, C.D., Silva Soley, B., De Mello-Sampayo, C., Cabrini, D.A., Correr, C.J., Otuki, M.F., 2016. Quantitative genotoxicity assays for analysis of medicinal plants: A systematic review. J. Ethnopharmacol.

Sreelatha, S., Padma, P.R., 2011. Modulatory effects of Moringa oleifera extracts against hydrogen peroxide-induced cytotoxicity and oxidative damage. Hum. Exp. Toxicol. 30, 1359–1368.

Stanhope, K.L., Havel, P.J., 2008. Fructose consumption: Potential mechanisms for its effects to increase visceral adiposity and induce dyslipidemia and insulin resistance. Curr. Opin. Lipidol.

Steyn, N.P., Myburgh, N.G., Nel, J.H., 2003. Evidence to support a food-based dietary guideline on sugar consumption in South Africa. Bull. World Health Organ. 81, 599–608.

Steyn, N.P., Temple, N.J., 2012. Evidence to support a food-based dietary guideline on sugar consumption in South Africa. BMC Public Health 12, 1.

Stohs, S.J., Hartman, M.J., 2015. Review of the Safety and Efficacy of Moringa oleifera. Phyther. Res. 29, 796–804.

Stumvoll, M., Goldstein, B.J., van Haeften, T.W., 2005. Type 2 diabetes: principles of pathogenesis and therapy. Lancet (London, England) 365, 1333–46.

Subramaniam, S., Subramaniam, R., Rajapandian, S., Uthrapathi, S., Gnanamanickam, V.R., Dubey, G.P., 2011. Anti-atherogenic activity of ethanolic fraction of terminalia arjuna bark on hypercholesterolemic rabbits. Evidence-based Complement. Altern. Med. 2011.

Sun, S.Z., Empie, M.W., 2012. Fructose metabolism in humans--what isotopic tracer studies tell us. Nutr. Metab. 9, 1–15.

Tangvarasittichai, S., Pongthaisong, S., Tangvarasittichai, O., 2016. Tumor Necrosis Factor-A, Interleukin-6, C-Reactive Protein Levels and Insulin Resistance Associated with Type 2 Diabetes in Abdominal Obesity Women. Indian J. Clin. Biochem. 31, 68–74.

Tappy, L., Le, K.-A., 2010. Metabolic Effects of Fructose and the Worldwide Increase in Obesity. Physiol. Rev. 90, 23–46.

Teixeira, E.M.B., Carvalho, M.R.B., Neves, V.A., Silva, M.A., Arantes-Pereira, L., 2014. Chemical characteristics and fractionation of proteins from Moringa oleifera Lam. leaves. Food Chem. 147, 51–54.

Thulin, P., Rafter, I., Stockling, K., Tomkiewicz, C., Norjavaara, E., Aggerbeck, M., Hellmold, H., Ehrenborg, E., Andersson, U., Cotgreave, I., Glinghammar, B., 2008. PPARα regulates the hepatotoxic biomarker alanine aminotransferase (ALT1) gene expression in human hepatocytes. Toxicol. Appl. Pharmacol. 231, 1–9.

Titmuss, A.T., Srinivasan, S., 2016. Metabolic syndrome in children and adolescents: Old concepts in a young population. J. Paediatr. Child Health.

Toma, A., Deyno, S., 2014. Phytochemistry and pharmacological activities of moringa oliefera. Int. J. Pharmacogn. 1, 222–231.

Toriola, A.L. AL, Moselakgomo, V.V., Shaw, B.B., Goon, D.T.D., 2012. Overweight , obesity and underweight in rural black South African children. South African J. Clin. Nutr. 25, 57–61.

Trapani, L., Segatto, M., Pallottni, V., 2012. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic "power station." World J. Hepatol. 4, 184.

Tripathi, Parul, Tripathi, Prashant, Kashyap, L., Singh, V., 2007. The role of nitric oxide in inflammatory reactions. FEMS Immunol. Med. Microbiol. 51, 443–452.

Tune, J.D., Goodwill, A.G., Sassoon, D.J., Mather, K.J., 2017. Cardiovascular consequences of metabolic syndrome. Transl. Res. 183, 57–70.

Valasek, M.A., Clarke, S.L., Repa, J.J., 2007. Fenofibrate reduces intestinal cholesterol absorption via PPARα-dependent modulation of NPC1L1 expression in mouse. J. Lipid Res. 48, 2725–2735.

Verma, A.R., Vijayakumar, M., Mathela, C.S., Rao, C. V, 2009. In vitro and in vivo antioxidant properties of different fractions of Moringa oleifera leaves. Food Chem. Toxicol. 47, 2196–2201.

Vongsak, B., Sithisarn, P., Gritsanapan, W., 2013. Simultaneous HPLC Quantitative Analysis of Active Compounds in Leaves of Moringa oleifera Lam. J. Chromatogr. Sci. 52, 641–645.

Vos, M.B., Lavine, J.E., 2013. Dietary fructose in nonalcoholic fatty liver disease. Hepatology.

Wahba, I.M., Mak, R.H., 2007. Obesity and obesity-initiated metabolic syndrome: Mechanistic links to chronic kidney disease. Clin. J. Am. Soc. Nephrol. 2, 550–562.

Weiss, R., Bremer, A.A., Lustig, R.H., 2013. What is metabolic syndrome, and why are children getting it? Ann. N. Y. Acad. Sci. 1281, 123–140.

WHO, 2018. Obesity and overweight, World Health Organisation. http://www.who.int>News>Fact sheets>Detail.

Wilcox, G., 2005. Insulin and Insulin Resistance. Clin Biochem Rev 26, 19–39.

Wolf, A., Bray, G.A., Popkin, B.M., 2008. A short history of beverages and how our body treats them. Obes. Rev.

Wolf, G., Hamann, A., Han, D.C., Helmchen, U., Thaiss, F., Ziyadeh, F.N., Stahl, R.A.K., 1999. Leptin stimulates proliferation and TGF-β expression in renal glomerular endothelial cells: Potential role in glomerulosclerosis. Kidney Int. 56, 860–872.

Wooten, J.S., Nick, T.N., Seija, A., Poole, K.E., Stout, K.B., 2016. High-Fructose Intake Impairs the Hepatic Hypolipidemic Effects of a High-Fat Fish-Oil Diet in C57BL/6 Mice. J. Clin. Exp. Hepatol. 6, 265–274.

World Health Organization (WHO), 2002. WHO Traditional Medicine Strategy 2002-2005, World Health Organisation Geneva.

Yang, X., Li, Yang, Li, Yanda, Ren, X., Zhang, X., Hu, D., Gao, Y., Xing, Y., Shang, H., 2017. Oxidative stress-mediated atherosclerosis: Mechanisms and therapies. Front. Physiol. 8, 1–16.

Younossi, Z., Anstee, Q.M., Marietti, M., Hardy, T., Henry, L., Eslam, M., George, J., Bugianesi, E., 2017. Global burden of NAFLD and NASH: Trends, predictions, risk factors and prevention. Nat. Rev. Gastroenterol. Hepatol. 15, 11–20.

Zaku, S., Emmanuel, S., Tukur, A., Kabir, A., 2015. Moringa oleifera: An underutilized tree in Nigeria with amazing versatility: A review. African J. Food Sci. 9, 456–461.

Zvinorova, P.I., Lekhanya, L., Erlwanger, K., Chivandi, E., 2015. Dietary effects of Moringa oleifera leaf powder on growth, gastrointestinal morphometry and blood and liver metabolites in Sprague Dawley rats. J. Anim. Physiol. Anim. Nutr. (Berl). 99, 21–28.

APPENDICES

APPENDIX I: Ethics clearance certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2016/10/44/C

APPLICANT: Ms M Mapfumo

SCHOOL: School of Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Effects of ethanolic Moringa oleifera seed extract on high-cholesterol high-fructose diet fed Sprague Dawley (Rattus norvegicus) rats

Number and Species

50 X 21 day old Male Sprague Dawley rats and 50 X 21 day old female Sprague dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2016/10/25. This approval remains valid until 2019/01/24.
Unreported changes to the application may invalidate the clearance given by the AESC

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 31/01/2017

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 31.01.17

cc: Supervisor: Dr E Chivandi
Director: CAS

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Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Mapfumo Masiline (530525)

b. School and email address: School of Physiology. lmasiline@yahoo.com

c. Experiment to be modified / extended

	AESC NO		
Original AESC number	**2016**	**10**	**44C**
Other M&Es :			1

d. Project Title: Effects of ethanolic *Moringa oleifera* seed extract on high- cholesterol high-fructose diet fed growing Sprague Dawley rats.

		No.	Species
e.	Number and species of animals originally approved:	100	Sprague Dawley
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	104	
h.	Number of animals used to date:	104	

i. Specific modification / extension requested:

- Request for 50 Additional SD rats (24 males and 26 females) including the 4 extra males which have already been allocated to the study.

j. Motivation for modification / extension:

The high cholesterol diet negatively affected animal feed intake and weight difference between the experimental rats and control rats was more than 15%. In line with the end point for welfare intervention, I had to remove the animals from the high cholesterol diet. And they were returned to the CAS who will channel them for dissections.
The diet used to include metabolic dysfunction contained 1% cholesterol and the rats had access to 20% fructose solution. I now require 50 replacement experimental rats (4 have already been allocated to the study). The rats will replace the 50 that were returned to stock. They will however not receive cholesterol but will receive the 20% fructose which we have previously used.

Date:28/06/2017 Signature:

RECOMMENDATIONS: Approved. 50 additional rats as detailed above. The total now approved for the study is 150.

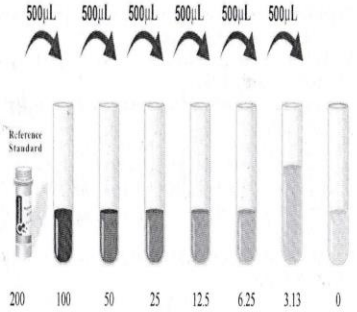
Date: 29 June 2017 Signature:

Chairman, AESC

APPENDIX II: Insulin ELISA protocol

This reconstitution produces a working solution of 200ng/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 200、100、50、25、12.5、6.25、3.13、0 ng/mL.

Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 200ng/mL working solution to the first tube and mix up to produce a 100ng/mL working solution. Pipette 500uL of the solution from former tube to the latter one in order according to this step. The illustration below is for reference. Note: the last tube is regarded as blank. Don't pipette solution to it from the former tube.



4. **Biotinylated Detection Ab working solution**: Calculate the required amount before experiment (100μL/well). In actual preparation, you should prepare 100~200μL more. Centrifuge the stock tube before use, dilute the 100×concentrated Biotinylated Detection Ab to 1×working solution by Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution**: Calculate the required amount before experiment (100μL/well). In actual preparation, you should prepare 100~200μL more. Dilute the 100×Concentrated HRP Conjugate to 1×working solution by HRP Conjugate Diluent.

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Assay procedure(A brief assay procedure is on the 11th page)

1. Add **Standard working solution** of different concentration to the first two columns: Each concentration of the solution is added into two wells side by side(100uL for each well). Add samples to other wells(100uL for each well). Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃. Note: solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible.
2. Remove the liquid of each well, don't wash. Immediately add 100μL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37℃.
3. Aspirate or decant the solution from each well, add 350μL of **wash buffer** to each well. Soak for 1~2 minutes and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times in total. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100μL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37℃.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 90μL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 minutes at 37℃. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30minutes.
7. Add 50μL of **Stop Solution** to each well. Note: the order to add stop solution should be the same as the substrate solution.
8. Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm.

Calculation of results

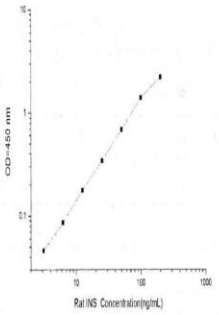
Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution.The actual concentration is the calculated concentration multiplied dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of actual assay performance(e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish standard curve for each test. Typical standard curve and data below is provided for reference only.

Concentration(ng/mL)	200	100	50	25	12.5	6.25	3.13	0
OD	2.227	1.435	0.796	0.453	0.245	0.167	0.125	0.079
Corrected OD	2.148	1.356	0.717	0.374	0.166	0.088	0.046	-



Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level Rat INS were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level Rat INS were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
mean(ng/mL)	10.4	24.1	80.4	10.9	22.1	74.3
Standard deviation	0.6	1.2	3.1	0.7	1	2.9
CV(%)	5.77	4.98	3.86	6.42	4.52	3.9

Recovery

The recovery of Rat INS spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	89-103	95
EDTA plasma (n=5)	88-104	95
Cell culture media (n=5)	92-107	100

Linearity

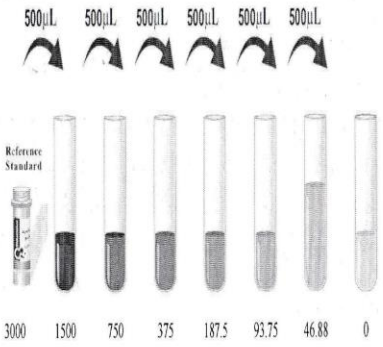
Samples were spiked with high concentrations of Rat INS and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma (n=5)	Cell culture media (n=5)
1:2	Range (%)	85-98	94-108	94-108
	Average (%)	91	100	100
1:4	Range (%)	100-114	81-91	96-109
	Average (%)	106	86	102
1:8	Range (%)	96-108	86-97	95-108
	Average (%)	102	91	102
1:16	Range (%)	94-108	82-93	97-108
	Average (%)	101	88	103

APPENDIX III: Adiponectin ELISA protocol

This reconstitution produces a working solution of 3000pg/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 3000、1500、750、375、187.5、93.75、46.88、0 pg/mL.

Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 3000pg/mL working solution to the first tube and mix up to produce a 1500pg/mL working solution. Pipette 500uL of the solution from former tube to the latter one in order according to this step. The illustration below is for reference. Note: the last tube is regarded as blank. Don't pipette solution to it from the former tube.



4. **Biotinylated Detection Ab working solution**: Calculate the required amount before experiment (100μL/well). In actual preparation, you should prepare 100~200μL more. Centrifuge the stock tube before use, dilute the 100×concentrated Biotinylated Detection Ab to 1×working solution by Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution**: Calculate the required amount before experiment (100μL/well). In actual preparation, you should prepare 100~200μL more. Dilute the 100×Concentrated HRP Conjugate to 1×working solution by HRP Conjugate Diluent.

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Assay procedure(A brief assay procedure is on the 11th page)

1. Add **Standard working solution** of different concentration to the first two columns: Each concentration of the solution is added into two wells side by side(100uL for each well). Add samples to other wells(100uL for each well). Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃. Note: solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible.
2. Remove the liquid of each well, don't wash. Immediately add 100μL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37℃.
3. Aspirate or decant the solution from each well, add 350μL of **wash buffer** to each well. Soak for 1~2 minutes and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times in total. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100μL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37℃.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 90μL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 minutes at 37℃. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30minutes.
7. Add 50μL of **Stop Solution** to each well. Note: the order to add stop solution should be the same as the substrate solution.
8. Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm.

Calculation of results

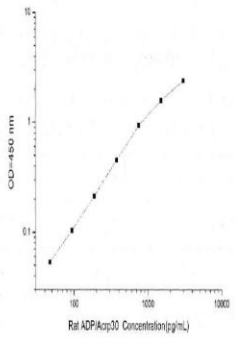
Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution.The actual concentration is the calculated concentration multiplied dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of actual assay performance(e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish standard curve for each test. Typical standard curve and data below is provided for reference only.

Concentration(pg/mL)	3000	1500	750	375	187.5	93.75	46.88	0
OD	2.528	1.599	0.927	0.521	0.248	0.167	0.116	0.063
Corrected OD	2.465	1.536	0.864	0.458	0.185	0.104	0.053	-



Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level Rat ADP/Acrp30 were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level Rat ADP/Acrp30 were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
mean(pg/mL)	148.5	342.6	1280.4	151.5	366.9	1345.9
Standard deviation	9.4	19.2	66.6	10.5	15	59.2
CV (%)	6.33	5.6	5.2	6.93	4.09	4.4

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Recovery

The recovery of Rat ADP/Acrp30 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	92-104	97
EDTA plasma (n=5)	94-105	100
Cell culture media (n=5)	90-107	97

Linearity

Samples were spiked with high concentrations of Rat ADP/Acrp30 and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma (n=5)	Cell culture media (n=5)
1:2	Range (%)	85-98	96-110	90-102
	Average (%)	92	103	97
1:4	Range (%)	97-112	81-94	97-110
	Average (%)	104	86	104
1:8	Range (%)	101-115	85-101	96-113
	Average (%)	108	92	104
1:16	Range (%)	97-110	80-94	94-106
	Average (%)	105	86	101