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EFFECTS OF HYDROETHANOLIC *OPUNTIA FICUS-INDICA* CLADODE EXTRACT ON HIGH-FRUCTOSE HIGH-FAT DIET-FED GROWING SPRAGUE-DAWLEY RATS

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

Johannesburg, 2019

DECLARATION

I, **Abigail Zamazulu Zulu,** declare that the dissertation hereby submitted to the University of Witwatersrand for the degree of Master of Science in Medicine (Physiology) has not been submitted previously by me or anybody for a degree at this or any other University. Also, this is my work in design and in execution. Related materials contained herein are duly acknowledged.



Abigail Zamazulu Zulu

Signed on the **20th** day of **April** 2020

DEDICATION

To my Family with Love

&

To my late Sister, Constance Sylvia Mgidi

1972-2010

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I would firstly like to offer my heartfelt gratitude and appreciation to my supervisors Dr Busisani Wiseman Lembede and Associate Professor Eliton Chivandi who supported, guided and encouraged me during the experimental phase of my study and dissertation write up. My supervisors gave me the autonomy to conceptualise ideas, solve problems and this helped me develop the capability to effectively work independently and collaboratively.

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I would secondly like to unaffectedly thank my loving husband Setagwa Peter Mahlo who has always been my source of strength, my pillar and always brings out the best in me and our son Seshego Thapelo Mahlo who is a blessing to us. To my parents Mr Wilson Boet Zulu and Mrs Sophie Kinah Zulu I will forever be grateful to you for the firm upbringing and for motivating my siblings and me to pursue and achieve anything that we envision in life. To my sister Lindiwe Jumaima Zulu and my brother Zwelibanzi Solomon Zulu, I could not have asked for better siblings; multiple thanks for always being there for me.

Lastly, I would like to give thanks to the Almighty God, who created all of us for a reason, may His Holy name always and forever be praised. Amen.

ABSTRACT

The consumption of diets rich in refined sugars and fats and lack of exercise are the main contributors to the global increase in the prevalence of obesity, metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD) and type-II-diabetes mellitus (T-II-DM). *Opuntia ficus-indica* cladode extract possesses antioxidant, anti-inflammatory, anti-obesogenic and antidiabetic properties. This study sought to determine the potential prophylactic effects of the hydroethanolic *Opuntia ficus-indica* cladode extract against high-fructose high-fat diet-induced metabolic derangements in growing rats modelling children fed obesogenic diets.

Ninety-eight 21-day-old weaned Sprague-Dawley rat pups (49 males; 49 females) were randomly allocated to one of five dietary groups and administered the following treatment regimens daily for 12 weeks: group I - standard rat chow (SRC) plain drinking water (PW) + plain gelatine cube (PC); group II - SRC + 2% (w/w) beef tallow (SRCB) + 25% (w/v) fructose solution (FS) + PC; group III- SRCB + FS + 100 mg/kg body mass fenofibrate in gelatine cube (FeC); group IV - SRCB + FS + low dose (50 mg/kg body mass) of *Opuntia ficus-indica* cladode extract in gelatine cubes (LOpC); group V - SRCB +FS + high dose (500 mg/kg body mass) of *Opuntia ficus-indica* cladode extract in gelatine cube (HOpC). After the 12-week feeding trial, the rats were subjected to oral glucose tolerance tests following an overnight fast. Immediately thereafter the rats were returned to their respective treatments, allowed a 48-hour recovery period following which they were fasted overnight, and their terminal body masses and fasting blood glucose concentrations were determined prior to euthanasia. Harvested plasma was used in the determination of triglycerides, total and HDL-cholesterol, insulin, TBARS and surrogate markers of liver and kidney function. Viscera macromorphometry, liver lipid content and histology were also determined.

The high-fructose high-fat diet caused ($p < 0.05$) increased liver lipid accretion in the rats but increased kidney mass in male rats only. Remarkably, the orally administered hydroethanolic *Opuntia ficus-indica* cladode extract and fenofibrate prevented the development of the high-fructose high-fat diet-induced fatty-liver disease in the male and female rats. Fenofibrate caused ($p < 0.05$) hepatomegaly, hyperglycaemia and increased ($p < 0.05$) plasma ALP activity in high-fructose high-fat diet-fed rats.

Oral administered of high and low dose of hydroethanolic *Opuntia ficus-indica* cladode extract protects growing rats against the development of high-fructose high-fat diet-induced fatty-liver disease without compromising liver and kidney function.

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LIST OF ABBREVIATIONS

α:	Alpha
β:	Beta
γ:	Gamma
AESC:	Animal Ethics Screening Committee
ALP:	Alkaline phosphatase
AUC:	Area under the curve
AST:	Aspartate aminotransferase
ANOVA:	Analysis of variance
BMI:	Body mass index
BUN:	Blood urea nitrogen
CAS:	Central animal service
CRP	C-reactive protein
CRTP-5	C1q/tumour necrosis factor-related protein-5
DOHaD	Developmental Origins of Health and Disease
DHAP	Dihydroxyacetone phosphate
ELISA:	Enzyme-linked immunosorbent assay
FeC:	Fenofibrate gelatine cube
FS:	20% w/v fructose water solution
GAE:	Gallic acid equivalent
GGT:	Gamma-glutamyl transferase
GLUT:	Glucose transporter
H&E:	Haematoxylin and eosin
HDL-chol:	High-density lipoprotein cholesterol
HFHF:	High-fructose high-fat diet
HOMA-IR:	Homeostatic model of insulin resistance
HOpC:	High dose of *Opuntia ficus-indica* cladode extract
IL-6:	Interleukin-6
LOpC:	Low dose of *Opuntia ficus-indica* cladode extract
LDL:	Low-density lipoprotein
LI:	Large intestine
MetS:	Metabolic syndrome
nd:	Not detected
NALFD:	Non-alcoholic fatty liver disease

NAS:	Non-alcoholic fatty liver disease activity score
NASH:	Non-alcoholic steatohepatitis
OGTT:	Oral glucose tolerance test
PC:	Plain gelatine cubes
PW:	Plain water
PPAR:	Peroxisome proliferator-activated receptor
SD:	Standard deviation
SRC:	Standard rat chow
SRCB:	Standard rat chow with 2% w/w beef tallow
TBARS:	Thiobarbituric acid reactive substances
Total-chol:	Total cholesterol
TNF:	Tumour necrosis factor
TGs:	Triglycerides
TZD:	Thiazolidinedione
T-II-DM:	Type 2 diabetes mellitus
VLDL:	Very low-density lipoprotein
w/w	weight/weight
w/v:	weight/volume
WHO:	World Health Organization

CHAPTER 1: INTRODUCTION

1.0 Preview of the dissertation

This dissertation comprises of five chapters. Chapter one introduces the problem of obesity in children. It gives key statistics on obesity and an analysis of the research done to date with regards to the problem. It then expresses the justification, aim, objectives and hypothesis of the study.

Chapter two critically explores and highlights the literature that is pertinent to the study. In chapter two, concepts of childhood obesity, metabolic syndrome, non-alcoholic fatty liver disease and ethnomedicines in managing metabolic disorders are appraised. The chapter gives a narrative on the prevalence of obesity as a risk factor for metabolic syndrome as well as various other associated health and or metabolic outcomes. Lastly, it also reviews the biological classification, chemical composition and the medicinal bioactivities of *O. ficus-indica.*

Chapter three begins by describing the source of *O. ficus-indica* and how the hydroethanolic extract was prepared. Thereafter the narration on the methods used to qualitatively and quantitatively determine the phytochemicals in the *O. ficus-indica* extract are given. The chapter then goes on to describe the experimental animals, preparation of dietary treatments, study design and terminal procedures. Chapter three ends by describing how the statistical analyses were conducted.

Chapter four presents the findings of the study.

Chapter five critically analyses and interprets the findings of the study in relation to current and relevant literature and draws the attention of the reader to the potential applications of the study findings.

Chapter six provides key conclusions and limitations of the study and then makes recommendations for future possible studies.

Chapter seven provides a list of sources that are cited in the dissertation.

1.1 Introduction

Obesity is defined as the excessive accumulation of fat that may result in adverse health outcomes (Hebebrand and Hinney 2009). In 2016, approximately 41 million children under the age of 5 years old were obese globally and 35 million of these children were in developing countries, with 26% being from South Africa (Hebebrand and Hinney 2009). Obesity is associated with adverse health outcomes inclusive of MetS, NAFLD and T-II-DM and cardiovascular diseases (Farrell *et al.,* 2018). Metabolic syndrome refers to the cocktail manifestation of risk factors inclusive of visceral obesity, hyperglycaemia, insulin resistance and dyslipidaemia (Chalasani et al. 2018). These risk factors increase the risk of developing T-II-DM and cardiovascular diseases (Chalasani et al. 2018; Monyeki et al. 2015). Type-II-diabetes mellitus and cardiovascular diseases are amongst the leading causes of mortality worldwide (Murphy et al. 2012). Non-alcoholic fatty liver disease, which is regarded as the hepatic manifestation of MetS, results from the excessive infiltration and accumulation of lipids in the liver which is not induced by alcohol consumption (Lonardo *et al.*, 2015; Farrell *et al.,* 2018). The main contributors to the global increase in the prevalence of obesity and its associated adverse metabolic outcomes (MetS, NAFLD and T-II-DM) include physical inactivity and increased consumption of diets rich in refined sugars and fats (Farrell *et al.,* 2018). Whereas fructose, which is twice sweeter than glucose, is commonly used as a sweetener in various foods and beverages (Lim et al. 2019), lipids are found in various animal food products such as meat, dairy products and eggs (Czech 2017).

Animal and human studies have shown that the consumption of diets that have high fructose and fat content results in obesity, MetS and NAFLD (Laakso and Kuusisto 2014; Lim et al. 2019). The metabolism of fructose, which occurs primarily in the liver, does not stimulate insulin secretion and bypasses key rate-limiting steps in glycolysis (Ferraris *et al*., 2018). Thus the metabolism of fructose results in increased hepatic *de novo* lipogenesis, dyslipidaemia and visceral obesity (Myers 2017). The consumption of high-fat diets decreases hepatic β-oxidation but increases hepatic triglyceride and VLDL synthesis (Borén et al. 2013). Based on the aforesaid the consequences of consuming diets rich in both fructose and fat include increased hepatic lipid accretion, dyslipidaemia as well as visceral obesity and subsequently the manifestation of MetS and NAFLD. Visceral obesity, which causes oxidative stress, is associated with increased secretion of inflammatory cytokines: TNFα and IL-6 from adipocytes (Takaki *et al*., 2013). The combination of inflammatory cytokines and increased oxidative stress lead to hyperglycaemia, insulin resistance, dyslipidaemia and organ damage (Orasanu and Plutzky 2009).

Conventional pharmacological agents such as statins, thiazolidinedione and fenofibrate can be used to treat or manage specific features of obesity, MetS and NAFLD (Ghodsi et al. 2017). However, these pharmacological agents are costly and inaccessible to the majority of the global population (Magrini et al. 2015). These synthetic pharmacological agents are also associated with adverse side effects (Merone and McDermott 2017). Additionally, most pharmacological agents have a single or specific pharmacological target and or action. However, obesity, MetS and NAFLD typically manifest with multifactorial pathologies (Pereira et al. 2017). Thus, as a consequence of the above-mentioned limitations of the conventional pharmacological agents, majority of the global population favours the use of natural plant-derived ethnomedicines that typically have multiple pharmacological actions (Sandhar et al. 2011). Natural plant-derived ethnomedicines are perceived as having a lower risk of causing side effects (Souza and Hawkins 2017). Nonetheless, the efficacy and toxicity of most natural plant-derived ethnomedicines have not been scientifically validated (Dutra et al. 2016). Thus, research aimed at interrogating the pharmacological activities of natural plant-derived ethnomedicines such as *O. ficus-indica* is essential for the development and promotion of the efficacious and safe use of ethnomedicines (M. Kaur 2012).

O. ficus-indica is an ethnomedicine that is known to possess multi-bioactive phytochemicals such as saponins, alkaloids, flavonoids, phenolics, tannins and terpenoids which confers it with medicinally beneficial effects (Féboli et al. 2016; Souza 2014). The phytochemicals and minerals in the cladodes of *O. ficus-indica* possess antioxidant, anti-inflammatory, antimicrobial, anti-obesogenic and antidiabetic properties (Boutakiout et al. 2018). *O. ficus-indica* has been demonstrated to attenuate hyperglycaemia, hepatomegaly, hypertriglyceridemia and hepatocyte injury in streptozotocin (STZ)-diabetic rats and in obese Zucker rats (Morán-Ramos, 2012; Nuñez-López *et al.*, 2013). In T-II-DM patients, the oral administration of 500 mg/kg body mass of aqueous *Opuntia ficus-indica* cladode extract attenuated hyperglycaemia (Frati *et al.*, 1990; Alizade and Azadbakht, 2016). Nuñez-López et al. (2013) demonstrated that oral administration of 50 mg/kg body mass of *O. ficus-indica* cladode flour for five days attenuated hyperglycaemia in STZ-diabetic rats. In obese Zucker rats, the consumption of 4% (w/w) of *O. ficus-indica* cladode powder in feed for 7 weeks resulted in reduced plasma triglyceride concentration, attenuated hepatomegaly and reduced the plasma activities of alanine and aspartate aminotransferases; which are surrogate markers of hepatocyte injury (Morán-Ramos 2012). The mechanisms by which *O. ficus-indica* cladode extracts exert their antidiabetic and anti-obesogenic effects include inhibition of glucose absorption from the gastrointestinal tract, suppression of hepatic gluconeogenesis, promotion

of hepatic lipid breakdown and the improvement of tissues' sensitivity to insulin (Morán-Ramos, 2012; Nuñez-López *et al.*, 2013).

1.2 Justification of the study

Majority of studies showing the antidiabetic and anti-obesity effects of *O. ficus-indica* cladode extracts have used them as interventions for treatment or management in adult chemically-induced diabetic rat models (Gouws et al. 2019). However, there is a growing problem of diet-induced obesity in children which cannot be neglected and it is always best to target prevention than treatment (Farpour-Lambert et al. 2015). Additionally, a large proportion of animal studies have a bias for using male animal models (Will et al. 2017). Nonetheless, empirical evidence indicates that males and females can respond differently to dietary and medicinal interventions (Bidmon and Terlutter 2015). Thus, there is a need for studies to evaluate potential prophylactic interventions for diet-induced obesity, MetS and NAFLD using growing male and female rats modelling children fed obesogenic diets. Therefore, the current study aimed to determine whether hydroethanolic (70%) *O. ficus-indica* cladode extract can protect against high-fructose high-fat diet-induced metabolic derangements in growing rats modelling children fed obesogenic diets.

1.3 Aims and objectives

The aim of the first experiment of the study was to quantitatively determine the total phenolic content and qualitatively determine the other phytochemical constituents of the hydroethanolic *O. ficus-indica* cladode extract.

The second experiment of the study aimed to evaluate the potential of hydroethanolic *O. ficus-indica* cladode extract to protect growing Sprague-Dawley rats against high-fructose high-fat diet-induced adverse metabolic and or health outcomes by specifically determining its effect on:

a. body mass and long bone indices.
b. tolerance to an oral glucose load.
c. indirect markers of peroxidation [thiobarbituric acid reactive substances (TBARS)].
d. blood metabolic substrates (glucose, triglycerides, HDL-cholesterol and total cholesterol) concentration.
e. insulin, a metabolism-regulating hormone and insulin resistance (HOMA-IR).

f. the mass of the liver, kidney, visceral and epididymal fat.

g. NAFLD parameters [liver lipid content, histo-morphometry and surrogate markers of liver function enzymes (plasma ALT and AST activity)].

h. surrogate markers (plasma creatinine and blood urea nitrogen) of kidney function.

1.4 Hypothesis

H_0: Oral administration of hydroethanolic *O. ficus-indica* cladode extract has no effect on the health and metabolic outcomes of growing Sprague-Dawley rats fed a high-fructose high-fat diet

H_1: Oral administration of hydroethanolic *O. ficus-indica* cladode extract has an effect on the health and metabolic outcomes of growing Sprague-Dawley rats fed a high-fructose high-fat diet.

The next chapter (chapter 2) provides an extensive analysis of the literature relevant to the study, with a particular focus on childhood obesity, MetS, and NAFLD. The chapter concludes by giving key information regarding the use of ethnomedicines in managing metabolic disorders and on the reported health-beneficial bioactivities of *O. ficus-indica* cladode extracts.

CHAPTER 2: LITERATURE REVIEW

2.0 Obesity

Globally, the number of obese children between the ages of 5 and 19 years has risen ten-fold in the past four decades (WHO 2017). Research evidence suggests that obese children are more likely to remain obese throughout their adult life (Park et al. 2013). It is estimated that, globally, obesity and its related complications account for 20% of global healthcare spending (Monyeki et al. 2015). The global increase in obesity has been attributed to the adoption of sedentary lifestyles and the consumption of obesogenic western-style diets (Hu et al. 2012). The obesogenic western-style diets are known to be fat-saturated and contain processed sugars (Reilly 2006).

In adults, obesity is commonly diagnosed and categorised using body mass index (BMI) which is a non-invasive and cost-effective tool. Body mass index is computed as weight (kg) divided by height (m^2) (Farrell *et al.,* 2018). In adults, a BMI of less than 18.5 is indicative of underweight; a BMI greater than 25 indicates overweight and a BMI greater than 30 indicates obesity (WHO 2015). In children under the age of 5, weight-for-height z-scores are used to diagnose and categorise obesity (H. Yang et al. 2017). While a weight-for-height z-score that is greater than two standard deviations above WHO child growth standards median reflects overweight; a weight-for-height z-score greater than three standard deviations above the WHO child growth standards median indicates obesity (de Onis and Woynarowska 2009). However, the limitation of BMI and weight-for-height z-score is that they do not provide an indication of how the adipose tissue is distributed in the body (Castro et al. 2017). Android obesity, characterised by central or visceral fat accumulation, is the most perilous form of obesity (Kawser Hossain et al. 2016). It increases the risk of developing MetS, T-II-DM and NAFLD (Farrell *et al.,* 2018). Visceral obesity is assessed by measuring waist circumference and or computing hip:waist circumference (Castro et al. 2017). Studies have shown that waist circumference is an accurate and integral predictor of childhood risk of developing metabolic syndrome (H. Yang et al. 2017).

2.1 Metabolic syndrome

Worldwide it is estimated that one in every four people have MetS (Saklayen 2018). The increasing prevalence of MetS worldwide has been attributed to the obesity epidemic (Farrell *et al.,* 2018). Metabolic syndrome is defined as the cluster manifestation of cardio-metabolic conditions which increase the risk of developing T-II-DM and cardiovascular disease by four-fold (Bell *et al.,* 2014). The cardio-metabolic conditions include visceral obesity, glucose

intolerance, hyperglycaemia, hyperinsulinemia, insulin resistance, dyslipidaemia, NAFLD and hypertension (Kassi et al. 2011; Lonardo et al. 2015).

Visceral obesity increases the production of pro-inflammatory adipokines (IL-6, TNF-α and CRP) and suppresses the production of anti-inflammatory adipokine (Srinivasan et al. 2019). Pro-inflammatory cytokines promote lipolysis in visceral adipose tissue and this consequently results in dyslipidaemia (Divella et al. 2019). Additionally, pro-inflammatory adipokines inhibit GLUT-4 coding mRNA, as well as the phosphorylation of the insulin receptor substrate protein-1 (Srinivasan et al. 2019) thus causing hyperglycaemia and insulin resistance (Wensveen et al. 2015).

Insulin resistance refers to the tissues' inability to potently respond to the effects of insulin (Henriksen 2019). The main physiological effects of insulin include increasing glucose uptake and the suppression of hepatic gluconeogenesis, inhibition of lipolysis and stimulation of lipogenesis (Laakso and Kuusisto 2014). Thus the main consequence of insulin resistance is hyperglycaemia and dyslipidaemia (Nolan et al. 2015). In adipose tissue, insulin resistance causes increased lipolysis, which leads to an influx of lipids (glycerides and free fatty acids) into circulation thus causing dyslipidaemia (Sears and Perry 2015). Cooperatively, hyperglycaemia and increased free fatty acids in circulation will stimulate the secretion of insulin (Laakso and Kuusisto 2014) and this could result in hyperinsulinemia. The hyperinsulinemia can exacerbate insulin resistance by desensitising insulin receptors (DeFronzo and Tripathy 2009).

Dyslipidaemia is characterised by abnormal blood lipid concentrations (Badimon and Chiva-Blanch 2019). It encompasses hypertriglyceridemia, hypercholesterolemia, hyper-low density lipoprotein (LDL)-cholesterolemia and hypo-high density lipoprotein (HDL)-cholesterolemia (Badimon and Chiva-Blanch 2019). It (dyslipidaemia) is associated with the formation of plaques in blood vessels and increased oxidative stress which subsequently leads to atherosclerosis (Lin et al. 2019). Atherosclerosis is a condition where lipid plaques form inside blood vessels resulting in their (vessels) stiffening and narrowing and the onset of hypertension (Rafieian-Kopaei et al. 2014). Importantly, dyslipidaemia can result in the infiltration and accumulation of lipids in the liver (Lin et al. 2019).

2.2 Non-alcoholic fatty liver disease

It is estimated that NAFLD affects 30% of the global population (Younossi *et al.*, 2016; Ofosu *et al.*, 2018). The highest NAFLD prevalence has been reported to be in the Middle East (31.79%) and South America (30.45%) (Ofosu *et al.*, 2018). Africa is reported to have the lowest prevalence of NALFD (13.48%) (Younossi et al. 2016). Non-alcoholic fatty liver disease is to a condition whereby lipids infiltrate and accumulate in the liver without any excessive alcohol consumption or use of steatotic medications (Giorgio et al. 2013). Under normal physiological conditions, total liver lipid content is expected to be approximately five percent of total wet liver mass (M.-X. Xu et al. 2019). The excess accumulation of lipid in the liver leads to increased lipid peroxidation and consequently increased production of reactive oxidative species (Khan and Newsome 2016). The reactive oxidative species damage hepatocyte cell membranes and activate the inflammatory response (Chalasani et al. 2018). Activation of the inflammatory response causes the NAFLD to advance to non-alcoholic steatohepatitis (NASH) (M.-X. Xu et al. 2019). Non-alcoholic steatohepatitis that is characterised by excessive hepatic lipid accumulation, inflammation and hepatocyte degeneration (Chalasani et al. 2018) can progress to cirrhosis and liver failure (Khan and Newsome 2016). Non-alcoholic fatty liver disease can be diagnosed using NAFLD activity score (NAS), Brunt's criteria of steatosis and ultrasound scanning (Fabbrini *et al.*, 2009). The progression and severity of NAFLD are assessed using the semi-qualitative, non-alcoholic fatty liver disease activity score criteria (Loria et al. 2010).

2.3 Complication of metabolic syndrome: Type-II-diabetes mellitus

Type-II-diabetes mellitus refers to a metabolic disorder characterised by hyperglycaemia and insulin resistance (Canivell and Gomis 2014). It (T-II-DM) is associated with obesity, MetS and cardiovascular disease (Goedeke *et al.*, 2019). The manifestation of T-II-DM can occur as a result of reduced insulin production and secretion by pancreatic β-cells and or decreased tissues responsiveness to insulin (Canivell and Gomis 2014). Globally, the prevalence of diabetes among adults over the age of 18 years has been reported to range from 4.7% - 8.5 % with the number of people with diabetes being 422 million in 2014 (WHO Global Report 2016). However, an estimated 1.6 million deaths were directly caused by diabetes in 2016 (WHO Global Report 2016). Therefore, studies interrogating the aetiology, pathogenesis and pathophysiology of metabolic disorders such as obesity; MetS, NAFLD and T-II-DM are of great value to the wellbeing of society.

2.4 Models of metabolic disorders

Metabolic disorders inclusive of obesity, MetS, NAFLD and T-II-DM are a major public health problem and cause approximately 28% of deaths globally (Saklayen 2018). Thus, in recent years, there has been an increasing interest in developing animal models that can passably mimic all the aspects of the human metabolic disorder pathophysiology. The various models that are used to study metabolic disorder include hormonally-, chemically-, genetically- and diet-induced rat models as well as metabolic programming models (Suman et al. 2016).

2.4.1 Genetically-induced models

Genetic animal models are appropriate for studying the pathogenesis and pathophysiology of metabolic disorders that have genetic aetiology (H. Xu et al. 2018). The leptin-deficient mice (*ob/ob*), leptin receptor-deficient mice (*db/db*) Zucker fatty rats (ZF), Zucker diabetic fatty rats (ZDF) are among the most commonly used genetic animal models (Suman et al. 2016).

2.4.2 Chemically-induced models

Alloxan and streptozotocin (STZ) are glucose analogues that enter pancreatic β-cells via the GLUT2 transporters (Lenzen 2008). Once inside, these glucose analogues damage the pancreatic β-cells and thus reduce and or impair insulin production and secretion (Lenzen 2008). Therefore, chemically-induced animal models are appropriate for studies that focus on T-I-diabetes mellitus and its associated complications.

2.4.3 Metabolic programming models

The metabolic programming models of metabolic disorders are premised on the Developmental Origins of Health and Disease (DOHaD) hypothesis. The DOHaD hypothesis of metabolic programming states that suboptimal stimuli in the critical early developmental periods of life can increase the propensity or resistance to develop metabolic disorders (Wadhwa et al. 2009; Mandy and Nyirenda 2018). Suboptimal nutrition (over and undernutrition) in the early developmental periods of life can result in epigenetic alterations that can temporarily or permanently change physiological function and disease risk (Mandy and Nyirenda 2018). The metabolic programming models are appropriate for interrogating the foetal and or neonatal aetiology of metabolic disorders.

2.4.4 Diet-induced models of metabolic disorders

The consumption of unhealthy obesogenic diets that are saturated with carbohydrates and fats has been identified as a key contributor to the obesity, NAFLD, MetS and T-II-DM epidemics (Popkin 2015; Panchal and Brown 2011). Thus, there is a growing need for research that investigates the pathology and pathophysiology of diet-induced metabolic disorders. The most commonly used animal diet-induced models of metabolic disorders include high-fructose, high-fat, high-fructose high-fat, or high-sucrose high-fat diets (Fujita and Maki 2016). Diet-induced models of metabolic disorders are commended for their ability to model metabolic disorders in a manner that truly reflects the aetiology in the global population (Panchal et al. 2011).

2.5 High-fructose high-fat diets and metabolic disorders

Fructose is a monosaccharide that occurs naturally in fruits, vegetables and honey (Bantle 2006). It is commonly used as a sweetener in various foodstuff and beverages, especially soft-drinks (Tappy and Lê 2015). In the jejunum, it is passively absorbed into the enterocytes through GLUT5 facilitated diffusion (Ferraris *et al.*, 2018). Fructose is transported out of the enterocyte, on the basal membrane, into the circulation via GLUT2 facilitated diffusion (Goodman 2010). Once the fructose is in circulation, it is transported to the liver via the hepatic vein where it is then metabolised (Abdelmalek et al. 2012). In the liver, it is first converted into fructose-1-phosphate by the enzyme fructokinase (Ferraris *et al.*, 2018). Fructose-1-phosphate is then split into two 3-carbon molecules, namely glyceraldehyde and dihydroxyacetone phosphate (DHAP) by aldolase enzyme (Douard and Ferraris 2008). Dihydroxyacetone phosphate can then enter the glycolytic pathway and undergo glycolysis (Goodman 2010). The glyceraldehyde gets converted into glyceraldehyde-3-phosphate (an isomer of DHAP), which undergoes a series of reaction to form acetyl-CoA (Ferraris et al. 2018). The acetyl-CoA can be converted in citrate (Goodman 2010). In glucose metabolism (glycolysis), increased acetyl-CoA, citrate and ATP inhibit phosphofructokinase, a major rate-limiting enzyme in glycolysis (Goodman 2010). The phosphofructokinase rate-limiting step is critical as it limits the amount of glucose that enters the lipogenic pathways and gets converted to triglycerides (de Sousa Rodrigues et al. 2017). The metabolism of fructose bypasses the phosphofructokinase rate-limiting step and thus results in increased *de novo* triglyceride synthesis (Ferraris et al. 2018). The triglycerides are stored in the liver and or packaged into VLDLs and transported to visceral adipose tissue where they are stored.

Therefore the consumption of diets rich in fructose can result in the manifestation NAFLD and visceral obesity as well as the associated complications (de Sousa Rodrigues et al. 2017).

Lipids are a group of organic compounds comprising of fats, oils, steroid hormones, and certain components of membranes (Sears and Perry 2015). In the small intestines, lipids are emulsified to small fat droplets by bile acids (Lonardo et al. 2015) and bile salts. The fat droplets are then digested by pancreatic lipases to form free fatty acids and monoglycerides (Montgomery and Turner 2015). The free fatty acids are then packaged into micelles by bile salts and absorbed into the enterocytes of the small intestine through passive diffusion (Sears and Perry 2015). Once inside the enterocyte, the free fatty acids and monoglycerides enter the endoplasmic reticulum (Lonardo et al. 2015) they are resynthesized into triglycerides (Sears and Perry 2015). The triglycerides are then packaged into chylomicrons (Vergès 2015) that then diffuse out of the basolateral membrane of the enterocyte into the lacteal system (Ueshima et al. 2005). The lacteal system transports the lipids into the circulation (Sears and Perry 2015) and then the lipids are transported to various tissues in the body including the liver, adipose and muscle where they are stored (Montgomery and Turner 2015). Thus, the consumption of diets saturated with lipids can lead to the manifestation of obesity, NAFLD and MetS (Francisqueti et al. 2017).

Several studies have reported that feeding high-fructose high-fat diet to rats results in the manifestation of metabolic disorders that closely mimic the metabolic pathologies in humans (Sekar et al. 2017; Francisqueti et al. 2017).

2.6 Interventions against metabolic disorders

Interventions against metabolic disorders include lifestyle changes, synthetic pharmacological agents and plant-derived ethnomedicines. Lifestyle change interventions include increasing physical activity and the consumption of healthy, low-calorie diets. However, one of the biggest challenges with lifestyle change interventions is the lack of compliance and the monetary and time costs of adopting healthy lifestyle habits. Currently, there is no standard treatment regimen for obesity, MetS and NAFLD (Madrigal-Santillán *et al.*, 2014). However, synthetic pharmacological agents, inclusive of fenofibrate, statins and thiazolidinedione are used to target specific features and or complications of obesity, MetS and NAFLD (Madrigal-Santillán et al. 2014).

2.6.1 Fenofibrate

Fenofibrate, a peroxisomal proliferator molecule, is a commonly used antihyperlipidaemic pharmacological agent (Mozundar and Liguori 2011; Israelian-Konaraki and Reaven 2005). Peroxisomal proliferators such as fenofibrate activate the peroxisome proliferator-activated receptor (PPAR)-α and modulate genes that are involved in lipid metabolism (Ueshima et al. 2005). The activation of PPAR-α upregulates fatty acid oxidation (Rakhshandehroo *et al.*, 2010; Rigano *et al.*, 2017). Fenofibrate has also been shown to inhibit the absorption of cholesterol in the small intestine (Valasek *et al.*, 2007). Several studies have reported that fenofibrate attenuates certain aspects of diet-induced obesity, MetS and NAFLD in rats fed obesogenic diets (Valasek *et al.*, 2007; Bray *et al.*, 2016; Rigano *et al.*, 2017).

Despite the availability of several synthetic pharmacological agents that could be used as therapeutic agents for obesity, MetS and NAFLD; there are several major drawbacks. Typically, synthetic pharmacological agents are expensive, associated with side-effects and have a single (specific) pharmacological action. This becomes a drawback, especially considering that obesity, MetS and NAFLD are multi-faceted diseases that can affect multiple physiological systems (Mozundar and Liguori 2011). Thus, there is a need to find complementary and alternative interventions such as plant-derived ethnomedicines that have multiple health-beneficial bioactivities.

2.7 Plant-derived ethnomedicines

As a consequence of the global increase in the prevalence of metabolic disorders, there has been a growing interest in plant-derived ethnomedicines that can be used to prevent, treat or manage metabolic disorders (Dutra et al. 2016). A significant proportion (approximately 80%) of the global population prefers to use plant-derived ethnomedicines to treat systemic ailments (Fyhrquist et al. 2002). Plant-derived ethnomedicines contain multiple phytochemicals and possess multiple pharmacological activities (Atanasov et al. 2015). Additionally, plant-derived ethnomedicines are natural and thus are perceived as having minimal to no risk of causing side effects (Dutra et al. 2016). Nonetheless, the efficacy and toxicity of most natural plant-derived ethnomedicines have not been scientifically validated (Souza and Hawkins 2017). Thus studies that focus on interrogating the pharmacological activities and potential toxicity of natural plant-derived ethnomedicines are essential for the development of efficacious and safe ethnomedicinal products (Heinrich and Jäger 2015).

2.8 Opuntia ficus-indica

Opuntia ficus-indica (L.) Mill. is commonly used for various nutritional and medicinal purposes. It is commonly referred to as the prickly pear (English), palma forrageira (Mexican), barbary fig (English), dithoro (Sepedi), idorofiya (isiNdebele), turksvy (Afrikaans) and umthelekisi (isiZulu) (Sugawara and Nikaido 2014; Féboli et al. 2016). *Opuntia ficus-indica* has been cultivated from pre-Columbian times in Mesoamerica and it is thus almost impossible to locate the exact origin of the species (Féboli et al. 2016). Nonetheless, most ethnobotanists concur that *O. ficus-indica* probably originated in the Central Mexican valley (Zimmermann 2017). Currently, *O. ficus-indica* is cultivated throughout the world.

2.8.1 Botanical description

Opuntia ficus-indica is a large trunk-forming segmented cactus plant which can reach a height of 5-7m with a crown of over 3m in diameter and a trunk up to 1m in diameter (Loflin and Loflin 2009; El-Mostafa et al. 2014). The *O. ficus-indica* plant is made up of the fruits, flowers, roots and the vegetative part (cladodes) (Feugang 2006). Its fruits, also known as prickly pears, are oblong, green when immature and they turn yellow, orange-red or purple in colour when ripe (Chauhan et al. 2010). Yellow-orange, cup-shaped flowers form at the tip end of the perimeter of the terminal cladodes (Figure 1.1) (El-Mostafa et al. 2014). The succulent green or blue-green vegetative parts, which are also referred to as the pads, joints, or cladodes, are ovoid and elongated (Féboli et al. 2016). Cladodes can be 18-60 cm long and 10-40 cm wide (Chauhan et al. 2010). The outer cladode, the chlorenchyma, may bear spines that are 1-2.5 cm and is fundamental for its photosynthesis (Loflin and Loflin 2009). Cladode inner parts are composed of white medullar parenchyma which is mainly responsible for storing water (Astello-García et al. 2015).

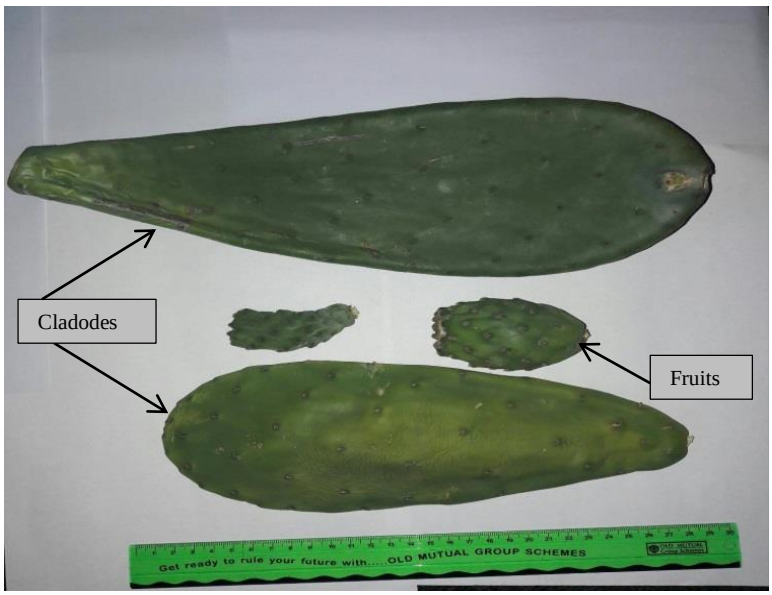

Figure 1.1: The cladode and raw fruit of *Opuntia ficus indica*

2.8.2 Taxonomic classification

Prickly pear belongs to Kingdom Plantae, Domain Eukaryota, Phylum Spermatophyta, Subphylum Angiospermae, Class Dicotyledonae, Order Caryophyllales, Family Cactaceae, Genus *Opuntia,* Species *ficus-indica* (A. and Q. luke Witt 2017).

2.8.3 Uses of Opuntia ficus-indica

Opuntia ficus-indica is extensively used as food, fodder and ethnomedicine (Butera et al. 2002; Rodríguez-Garcia et al. 2007). In Mexico and Italy, the *O. ficus-indica* cladode extracts are used traditionally to treat diabetes mellitus (M. Kaur 2012; Leem et al. 2016). The fruits, which are widely commercialised in many parts of the world, are eaten raw and are reported to have a high vitamin C content (de Sousa Rodrigues et al. 2017). In South America, the cladodes are cooked and eaten as a vegetable, in a recipe known as nopalitos (Souza 2014). *Opuntia ficus-indica* is an easily accessible and affordable alternative source of livestock feed (Desai and Desai 2015). However, its high water content may cause diarrhoea in livestock (Butera et al. 2002). Thus, it is not advisable for livestock to feed on a diet that is mainly made up of *O. ficus-indica* for long periods (Desai and Desai 2015). Industrially, *O. ficus-indica* is used to manufacture cosmetics, adhesives, fibre craft, paper, mucilage, ornamentation and flavouring agents (Souza 2014; de Sousa Rodrigues et al. 2017). Globally, *O. ficus-indica* is used in traditional ethnomedicine as a treatment for stomach ulcers, inflammation, hyperglycaemia, burn wounds, bronchoconstriction and indigestion (Souza and Hawkins 2017). In Mexico, the *O. ficus-indica* cladode extract is used traditionally to manage diabetes mellitus as well as treat hyperlipidaemia and obesity (Boutakiout et al., 2017).

2.8.4 Chemical composition of *Opuntia ficus-indica*

Opuntia ficus-indica pulp, skin and seeds are reported to have high water (>80%) content (Salim et al. 2009). The *O. ficus-indica* skin is made up of approximately 25% carbohydrate (Welegerima and Zemene 2017). *Opuntia ficus-indica* fruits contain carbohydrates (4-6%), protein (1-2%), minerals (1%) and high amount of vitamins, mainly A and C (Aragona et al. 2018).

The *O. ficus-indica* cladodes are reported to be a potential source of useful micronutrients and phytochemicals such as vitamins, flavonoids, tannins and saponins (Ennouri et al. 2014; Mena et al. 2018). The age of *O. ficus-indica* cladodes, environment, soil type and climate determine the variations in polyphenol contents (Souza 2014). The phenolic compounds are secondary metabolic products widely distributed in plants; they have many biological and pharmacological properties that could provide protection against chronic disease (Soldera-Silva et al. 2018; Senaphan et al. 2015).

2.8.4.1 Tannins

The cladodes of *O. ficus-indica* are rich in natural antioxidant compounds such as tannins (Morales et al. 2012). Tannins are water-soluble phenol derivatives naturally synthesized and accumulated by higher plants as secondary metabolic products, and they bind and precipitate proteins and carbohydrates (Jouany and Morgavi 2007; Szumacher-Strabel and Cielak 2012). Previous research has shown that tannins possess antidiabetic, anti-obesogenic, anti-inflammatory and antioxidant activities (Santos et al. 2013; Seo et al. 2018). In streptozotocin-diabetic rats, the administration of tannins has been shown to mitigate hyperglycaemia by improving tissue glucose uptake (Choudhury et al. 2018; Adinortey et al. 2019). Tannins improve glucose uptake by mediating the insulin-signalling pathways and by increasing GLUT-4 translocation in tissues (Adinortey et al. 2019; Kim et al. 2006). Moreover, tannins are reported to exert anti-hyperglycaemic effects by stimulating β-cell regeneration (Taher et al. 2016). According to Santos et al. (2013) tannins exert anti-inflammatory effects by inhibiting the production of IL-6, TNF-α and prostaglandin E_2. Studies have shown that tannins exert anti-obesity effect by reducing the expression of adipogenic and lipogenic transcription factors in white adipose tissue (Hong et al. 2017; Seo et al. 2018). Adipocyte differentiation is triggered by a set of interacting transcription factors such as the PPAR and CCAAT/enhancer binding protein (Zingarelli et al. 2003), whereas the lipogenesis entails induction of a lipogenic transcription factor such as sterol regulatory

element binding protein (SREBP1c), by PPARγ_2 (Softic *et al.*, 2016) SREBP1c, in turn, induces expression of lipolytic and lipogenic enzymes, which modulate fatty acid uptake and synthesis and thus adipocyte lipid accumulation (Hong et al. 2017).

2.8.4.2 Flavonoids

Opuntia ficus-indica cladodes contain unique flavonoids namely nicotiflorin, narcissin, isoquercetin and ferulic acid (Guevara-Figueroa et al. 2010). Flavonoids are known to possess various beneficial antioxidant, anti-obesogenic, antidiabetic and anti-inflammatory activities (Kawser Hossain et al. 2016; D. K. Yang and Kang 2018). According to Panche et al. (2016) flavonoids exert their antioxidant effects and protect tissues against reactive oxygen species by reducing free-radical formation and increasing scavenging of free radicals. Studies have shown that flavonoids attenuate hyperglycaemia, hypo-insulinemia and dyslipidaemia in streptozotocin-diabetic rats (Kaur *et al.*, 2018; Nazir *et al.*, 2018). It is reported that flavonoids reduce blood glucose concentration by suppressing gluconeogenic hepatic enzymes (such as AST and ALT), by protecting the pancreatic β-cells and by stimulating insulin secretion (Kawser Hossain et al. 2016; Al Hroob et al. 2018). In diet-induced obesity rat models, flavonoids have been shown to exert anti-obesity effects by enhancing insulin secretion and suppressing the expression of the lipogenic enzyme fatty acid synthase (Talebianpoor et al. 2019). Flavonoids have been shown to suppress the expression of lipogenic genes namely Sterol regulatory element-binding proteins (SREBP) and carbohydrate-responsive element-binding protein (ChREBP) (Softic *et al.*, 2016). Furthermore, flavonoids exert their antidiabetic activity by suppressing the expression of TNF-α coding mRNA in the visceral adipocytes (Tsuda et al. 2004). In a study conducted by Benayad *et al.* (2014), flavonoids derived from *O. ficus-indica* flowers exerted anti-inflammatory effects by inhibiting the production of pro-inflammatory eicosanoids and cytokines and through the suppression of histamine release by basophils.

2.8.4.3 Saponins

Saponins are secondary metabolites of glycosidic nature mainly derived from plant materials and have been used extensively in the drug industry due to their pharmacological properties (Podolak *et al.*, 2010; Cheok *et al.* 2014). They are known to exert an extensive range of pharmacological activities including anti-inflammatory, hypocholesterolaemia and hypoglycaemic (Sparg *et al.*, 2004; Sahu *et al.*, 2008). A study by Raymundo et al. (2011) showed that saponins exert their anti-inflammatory effects through reducing inflammatory

mediators (nitric oxide, IL-6, TNF-α and prostaglandin E_2). Saponins are known to exert anti-hyperglycaemic and anti-obesity effects by reducing insulin resistance and hyperglycaemia (Yang *et al.*, 2010; Lavle *et al.*, 2016; Elekofehinti *et al.*, 2017). *O. ficus-indica* cladode extracts are reported to possess strong antioxidant activities which contribute to their various therapeutic activities (Halmi et al. 2018).

2.9 Pharmacological activities of *Opuntia ficus-indica* cladodes

Cladodes are evidently known to be used for the treatment of gastritis, hyperglycaemia, hypercholesterolemia, arteriosclerosis, diabetes mellitus, prostatic hypertrophy and they also have anti-hyperlipidaemic action and immune-regulation function in the gastrointestinal tract (El-Mostafa et al. 2014).

2.9.1 Anti-diabetic activity

Opuntia ficus-indica cladode powder administration 3 times per day for 10 days at 100g/kg has been shown to reduce blood glucose and insulin concentration in T-II-DM patients (Gouws et al. 2019). The mechanisms by which the *O. ficus-indica* cladode powder exerts anti-hyperglycaemic effects include stimulation of pancreatic β-cell regeneration and insulin secretion and improvement of tissues' insulin sensitivity (Deldicque et al. 2013; Fransson et al. 2013).

2.9.2 Anti-inflammatory activity

A study by Antunes-Ricardo et al. (2015) revealed that in rats, *O. ficus-indica* cladode extract modulates the inflammatory response by suppressing COX-2 activity. Moreover, *in vitro* and *in vivo* studies have reported that *O. ficus-indica* cladode extracts exert anti-inflammatory effects by suppressing nitric oxide, IL-6, and TNF-α production (Chiricozzi et al. 2011; Hämäläinen et al. 2007). Research indicates that inflammation plays a crucial role in the manifestation and progression of metabolic disorders such as obesity, NAFLD and MetS (Lonardo et al. 2017). Thus, the anti-inflammatory activity of O. ficus-indica cladode extract makes it practical intervention for metabolic disarrays.

2.9.3 Antimicrobial Activities

A study by Welegerima and Zemene (2017) demonstrated that methanolic, ethanolic and aqueous *O. ficus-indica* cladode extracts had antimicrobial activity against *Vibrio cholerae* (Sánchez et al. 2016; Sánchez et al 2010). The gastro intestinal tract microbiota are involved in the digestion and absorption of various nutrients and substances in the gastrointestinal tract

(Wong et al. 2006; Valdes et al. 2018). It is well documented in literature that microbiota in the gastrointestinal tract play a crucial role in the development of metabolic disorders (Respondek et al. 2013; Turnbaugh et al. 2008). Thus, in the future, interventions for metabolic disorders are likely to target microbiota in the gastrointestinal tract. Hence the antimicrobial activity of *O. ficus-indica* cladode extract makes it a feasible intervention for metabolic disorders.

2.9.4 Antioxidant activity

A large body of knowledge confirms that metabolic disorders such as obesity, MetS and NAFLD are synonymous with oxidative stress and pro-inflammatory status (Mahjoub and Masrour-Roudsari 2012). Oxidative stress plays a crucial role in the manifestation, progression and severity of metabolic disorders (Vona et al. 2019). A study by El-Beltagi et al. (2019) showed that ethanol extract of the cladodes of *O. ficus-indica* exhibited a dose-dependent inhibition of linoleic acid oxidation in a thiocyanate assay system. In addition, *in vitro* assays have revealed that *O. ficus-indica* aqueous extract has dose-dependent free-radical scavenging activity (Antunes-Ricardo et al. 2015). The antioxidant activity of *O. ficus-indica* extracts is attributed to the high phenolic content of the extracts (Saleem et al. 2006; Francisqueti et al. 2017).

2.9.5 Anti-ulcer activity

Mucilage and pectin are major components of fresh *O. ficus-indica* cladodes (Falcon et al. 2010; Shetty et al. 2012). The oral administration of 1000 mg/kg of lyophilized *O. ficus-indica* cladodes protected Wistar rats against ethanol-induced ulcers (Galati et al. 2001). A study by Galati et al. (2001) showed that acute administration of lyophilized *O. ficus-indica* cladodes maintained the cytoarchitecture of the gastric mucosa in Sprague-Dawley rats. The *O. ficus-indica* cladode mucilage protects the gastrointestinal lining by forming a protective layer on the mucosa and thus preventing toxins and chemicals from penetrating the mucosa (Enza Maria Galati et al. 2003; Falcon et al. 2010). Additionally, *O. ficus-indica* cladode mucilage protects the gastric mucosa by stimulating increased gastric mucous production (Galati et al. 2003; Osuna-Martínez et al. 2014).

2.9.6 Hepatoprotective activity

A study using mice showed that *O. ficus-indica* cladode powder protects against organophosphate pesticide-induced liver toxicity and damage (Ncibi et al. 2008). The oral administration of *O. ficus-indica* aqueous cladode extract at 100 mg/kg for 60 days protected against lithium carbonate-induced hepatic damage in Wistar male rats (Saad et al. 2017).

Opuntia ficus-indica aqueous cladode extract exerts its hepatoprotective properties by inhibiting the production of pro-inflammatory mediators (Ncibi *et al.*, 2008; Saad *et al.*, 2017). The hepatoprotective activities of *O. ficus-indica* cladode extract make it a potential intervention for protection against diet-induced NAFLD.

2.10 Toxicology

It is estimated that less than 10% of plant-derived ethnomedicines in the world market have been scientifically tested or had their active compounds determined (Yun et al. 2017). The oral administration of aqueous *O. ficus-indica* cladode extract at 2g/kg for 3 months to rabbits did not elicit toxicity (Halmi et al. 2013). A study by Han et al. (2019) revealed that the oral administration of *O. ficus-indica* hydroethanolic cladode extract for a week at high-doses (500, 1000, and 2000 mg/kg per day) to adult rats did not cause toxicity. According to Han et al. (2019) *O. ficus-indica* cladode extracts do not cause adverse effects and thus can be used for medicinal purposes without causing toxicity.

CHAPTER 3: MATERIALS AND METHODS

3.0 Source of *Opuntia ficus-indica* cladodes

The cladodes of the *O. ficus-indica* were harvested in the North western region of Mpumalanga province, KwaMhlanga, South Africa (coordinates: 25°25'55"S 28°42'29"E) during the winter of 2017. The region is characterised by loamy soil and has an average annual rainfall of 767-878mm (Masereka et al. 2018) with mean annual temperature span from 10°C to 23°C (South African Weather Service. 1990). Voucher specimen *O. ficus-indica* cladodes were submitted to the University of the Witwatersrand's Herbarium for identification and authentication by a botanist. The voucher specimen was stored at the University of the Witwatersrand Herbarium under the reference number *J102442*.

3.1 Preparation of *Opuntia ficus-indica* extract

The *O. ficus-indica* cladodes were cleared of spines, rinsed with distilled water, chopped into 1cm × 1cm squares and freeze-dried (Labcon Freezone 4.5 freeze-dry system, Labcon, Kansas, MO, USA). Thereafter, the dried cladodes were ground into a fine powder using a blender (Multipro Compact, Kenwood, Roodepoort, South Africa). The powder (25g) was mixed with 500ml 70% ethanol and then the mixture was sonicated for an hour using a Misonix Ultrasonic Liquid processor ultra-sonicator (Millis, Massachusetts, USA). Immediately following sonication, the mixture was then filtered through filter paper (Whatmann®, No 1 size 9.0cm, England) under a vacuum. The resultant filtrate was then concentrated using a rota-evaporator (Labocon (Pty) Ltd, Krugersdorp, South Africa) at 78°C. The resultant concentrate was then dried in an oven (Salvis ®, Salvis Lab, Switzerland) overnight at 40°C. The dry extract was then stored in a sealed dark bottle at -4°C until use.

3.2 Determination of extract phytochemical composition

Qualitative tests were used to determine the presence or absence of alkaloids, flavonoids, terpenoids, saponins and tannins.

The presence of saponins was determined as described by Ezeonu and Ejikeme (2016). Briefly, 30ml distilled water was added into 300mg hydroethanolic *O. ficus-indica* cladode extract, boiled for 10 minutes in a water bath and filtered through filter paper (Whatman size 1). Thereafter the filtrate (10ml) was mixed with distilled water and mixed vigorously. Formation of a stable persistent froth indicated the presence of saponins.

Dragendorff's test was used to determine the presence of alkaloids. Briefly, 2mg of the extract was mixed with 5ml distilled water and then 5ml 2M hydrochloric acid was added to the mixture (Ezeonu and Ejikeme 2016). Thereafter, 1ml of Dragendorff's reagent was added to the mixture and the formation of an orange-red precipitate indicated the presence of alkaloids.

The presence of flavonoids was determined as previously described by Ju et al. (2018). In summary, 2g hydroethanolic *O. ficus-indica* cladode extract filtrate was mixed with 5ml 10% ammonium hydroxide in a test tube. Then 1ml concentrated sulphuric acid was added to the mixture and the presence of flavonoids was indicated by the development of a yellow colour.

To determine the presence of tannins, 2g of the extract was mixed with 30ml distilled water. Thereafter a few drops of 5% w/v iron chloride ($FeCl_3$) solution were added to the extract mixture (Ezeonu and Ejikeme 2016). The development of a green colour indicated the presence of hydrolysable tannins.

The presence of terpenoids in the extract was determined as described by Ayoola et al. (2008). In summary, 500mg of the extract was added into 2ml chloroform in a test tube following which 3ml of concentrated sulphuric acid was added to the mixture. The development of a reddish-brown colour in the interface layer indicated the presence of terpenoids.

The total phenolic content of hydroethanolic *O. ficus-indica* cladode extract was quantitatively determined as described by Rabeta and Faraniza (2013). Briefly, 1g of the extract was added into 50ml 80% methanol and then the mixture was shaken in an incubator for 24 hours at 30°C. The mixture was then centrifuged at 3200 x g for 20 minutes to obtain a supernatant. Two-hundred (200) microliters of the extract supernatant were then transferred into glass test tubes and 1ml of 1N Folin Ciocalteu phenol reagent was added to the mixture. The mixture was then vortexed and incubated for 5minutes at room temperature in the dark. Then 800 µL 7.5% anhydrous sodium chloride (Na_2CO_3) was added to each test tube, vortexed and incubated for an hour at room temperature in the dark. The absorbance of the mixtures was then measured at 765 nm with a spectrophotometer (DU 720, General purpose UV Spectrophotometer, Beckman Coulter). A standard curve was constructed using gallic acid (0-0.1 mg/mL). The total phenolic content of the extract was then expressed as gallic acid equivalents (GAE; mg/100g) of dried extract.

3.3 Ethical clearance and study site

The study, conducted in the Central Animal Service (CAS) unit, was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand, Johannesburg, South Africa (AESC number: 2017/09/61/B). All experimental procedures performed complied with the internationally accepted principles for laboratory animal use and care (Institute of Laboratory Animal Research Committee 2011).

3.4 Animals, feeding and housing

Ninety-eight 21-day-old weaned Sprague-Dawley rat pups (49 males; 49 females) were used for the study. The rats were housed individually in Perspex cages. The cages were lined with clean wood shavings for bedding which was changed twice a week. Room temperature was maintained at 24±2°C with a 12-hour light and dark cycle (lights on between 7 am-7 pm). Throughout the study, the rats had *ad libitum* access to rat chow and drinking fluid. The commercial rat chow used in this study was obtained from LabChef Research Nutrition at North West, South Africa). The rat chow had the following nutrient composition: crude protein 200 g/kg, crude oils and fats 50 g/kg, Linoleic acid 12 g/kg, crude fibre 40 g/kg, crude ash 70 g/kg, calcium 12 g/kg, phosphorus 7.5 g/kg and Vitamin A, D and E 16 000, 2 000 lU/kg and 100 mg/kg.

3.5 Diet, treatment doses and drug vehicle preparation

The standard rat chow pellets were ground to a meal using a hammer mill. For the experimental high-fat diet, 2% w/w of beef tallow (Sekar et al. 2017) was added to the standard rat chow meal and mixed thoroughly. Thereafter every kilogram of the standard rat chow meal or standard rat chow meal containing 2% w/w beef tallow was mixed with 400ml of water, reconstituted into biscuits and then dried in a ventilated oven at 37°C for 3 days.

The 25% fructose solution (w/v) was prepared by dissolving 250g of commercial fructose (Nature's Choice, Randvaal, South Africa) in 500ml of water and then making the solution into a litre. Red and blue food colouring [Robertson's Crimson food colouring, Retailer Brands (Pty) Ltd, South Africa] was added to the fructose solution and plain drinking water, respectively, to help with identification and differentiation of drinking fluids.

The hydroethanolic *O. ficus-indica* extract and fenofibrate doses used in the current study were similar to that used by Sun et al. (2015) and Rivera-Meza et al. (2017), respectively. The gelatine cubes used as vehicles for oral administration of hydroethanolic *O. ficus-indica* cladode extract and fenofibrate (Sigma-Aldrich, Modderfontein, Johannesburg, South Africa) were prepared as previously described by Kamerman et al. (2004).

3.6 Experimental design and treatment regimens

The ninety-eight (49 males, 49 females) 21-day-old weaned Sprague-Dawley rat pups were habituated for two-days prior to being randomly allocated to and administered the following treatment regimens daily for 12 weeks:

group I (10 males, 10 females) - served as the control group and received standard rat chow (SRC) + plain drinking water (PW) + plain gelatine cube (PC),

group II (9 males, 10 females) - served as the positive intervention for development of metabolic dysfunction and received standard rat chow with 2% (w/w) beef tallow (SRCB) + 25% (w/v) fructose solution (FS) + PC,

group III (10 males, 10 females) - served as the positive control intervention and received SRCB + FS + 100 mg/kg body mass fenofibrate in gelatine cube (FeC),

group IV (10 males, 9 females) - SRCB + FS + low dose (50 mg/kg body mass) of *O. ficus-indica* cladode extract in gelatine cubes (LOpC) and

group V (10 males, 10 females) - SRCB + FS+ high dose (500 mg/kg body mass) of *O. ficus-indica* cladode extract in gelatine cube (HOpC).

Throughout the experimental period, the rats were weighed twice per week using a Snowrex electronic balance (Model, Clover Scales, Johannesburg, South Africa). The body mass measurements allowed for the monitoring of growth and the maintenance of constant doses of the *O. ficus-indica* cladode extract and fenofibrate.

3.7 Determination of tolerance to an oral glucose load

After 12 weeks of treatments, each rat was fasted overnight prior to being subjected to an oral glucose tolerance test (OGTT). The fasting blood glucose concentration was determined (time interval 0) using a calibrated glucometer (Contour Plus, Bayer Corporation, Mishawaka, USA) as per manufacturer' instructions. Immediately following the determination of the fasting blood glucose concentration, each rat was then gavaged via orogastric intubation with 2g/kg body mass of sterile 50% (w/v) D-(+)-glucose solution (Sigma-Aldrich, Modderfontein,

Johannesburg, South Africa). Thereafter blood glucose concentrations were determined at time intervals 15, 30, 60 and 120 minutes post-gavage (Leng et al. 2004). Blood for determining glucose concentration was obtained via a pin-prick of the tail vein (Loxham et al. 2007).

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 then fasted overnight, and their terminal body masses and fasting blood glucose concentrations were then determined. The rats were then euthanised by intraperitoneal injection with an overdose of sodium pentobarbitone [Eutha-naze, Bayer (Pty) Ltd, Johannesburg, South Africa] at 200 mg/kg body mass following each rat was then carefully dissected and blood was collected via cardiac puncture using an 18G disposable needle and 10ml syringe. The blood from each rat was then transferred into heparinised blood tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The heparinised blood was centrifuged for 15 min at 5000 × g at 20°C (Hettich Centrifuge EBA-270, Tuttlingen, Germany) and the plasma was decanted into microtubes (Eppendorf, Hamburg, Germany) and stored at -20°C until use.

The abdomen of each rat was cut open by a midline incision. The liver, visceral and epididymal fat and kidneys were carefully dissected out. Thereafter the masses of each visceral organ were determined using an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). A sample of the liver was collected stored at -20ºC for determination of lipid content and the remainder of each liver samples was preserved in 10% phosphate-buffered formalin for histological analysis. The eviscerated carcasses were weighed to determine empty carcass mass.

3.9 Determination of blood parameters

3.9.1 Determination of insulin concentration

Plasma insulin concentration was determined using a rat insulin-specific enzyme-linked immunosorbent assay (ELISA) kit [Elabscience ®, Rat INS (Insulin) ELISA kit, Wuhan, Hubei Province, China] according to the manufacturer's directions. A detailed description of the protocol used is attached in Appendix 2. The assay used a sandwich-enzyme immunoassay principle utilising rat-specific insulin monoclonal antibody (sensitivity range:

0.31-20 ng/ml). The absorbencies of each sample were read at 450 nm using a plate reader (Multiskan Ascent, Lab system, model no 354, Helsinki, Finland). A standard curve was constructed using calibrator concentrations from which the insulin concentrations of the samples were determined.

3.9.2 Determination of plasma triglycerides, total and HDL-cholesterol

Plasma triglyceride and total cholesterol concentration were determined using an enzyme-linked calorimetric assay as per the manufacturer's instructions. A detailed description of the protocols used is attached in Appendix 3. The absorbencies of the samples were read at 510 nm using a spectrophotometer (DU 720, General purpose UV Spectrophotometer, Beckman Coulter).

High-density lipoprotein-cholesterol was determined using an enzyme-linked fluorescence HDL-cholesterol assay kit (Cell Biolabs, HDL and LDL/VLDL cholesterol assay kit, Cell Biolabs, Inc.) according to the manufacturer's guidelines. A detailed description of the protocol used is attached in Appendix 4. The fluorescence emissions of each sample were read using a microplate fluorescence reader (Synergy HT Fluorescence, USA) set for excitation between 530-570nm and for emission between 590-600nm. The relative fluorescence units of the calibrators were used to construct a standard curve from which the HDL-cholesterol concentrations of the samples were determined.

3.9.3 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances were quantitatively determined using a TBARS assay kit (R&D Systems, INC, biotech, USA) to determine the antioxidant status according to the manufacturer's instructions. The absorbencies of the samples were read using a microplate reader set at 540nm (Multiskan Ascent, Lab system, model no 354, Helsinki, Finland). A standard curve was then constructed using calibrator, and then concentrations of TBARS in the samples were determined.

3.9.4 Determination of surrogate markers liver and kidney

The plasma activities of alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) and concentrations of blood urea nitrogen and creatinine were determined using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions.

3.10 Determination of liver histo-morphometry

The preserved liver samples were routinely processed, embedded in paraffin wax, sectioned, and then stained with haematoxylin and eosin (H&E) (Zhou and Moore 2017). The H&E stained liver slides were then viewed under a light microscope and scored using the semi-quantitative non-alcoholic fatty liver disease activity score (Aly and Kleiner 2011). The NAFLD Activity Score (NAS), which is a semi-quantitative grading and scoring system, was used to grade the ballooning, lobular inflammation, and hepatic steatosis (Kleiner et al. 2005). NAS criteria used was as follows: *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; 3–4: uncertain; >5: probable or definite steatohepatitis (Kleiner *et al.* 2005).

3.11 Determination of liver lipid content

The total liver lipid content was determined using the Soxhlet with petroleum ether as the solvent as described by AOAC (2005; method number 920.39).

3.12 Statistical analysis

Parametric data are expressed as mean ± standard deviation and non-parametric data are expressed as median and range (min, max). All data were analysed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). Weekly body mass and oral glucose tolerance test data within a group were analysed using repeated-measures analysis of variance. A one-way analysis of variance was used to analyse multiple-group parametric data followed by a Tukey *post hoc* test for mean comparison. The Kruskal-Wallis test was used to analyse multiple-group non-parametric data followed by a Dunn's *post-hoc* test to compare medians. The level of significance was accepted at $p < 0.05$.

The model used for the analysis of variance for weekly body masses was:

$Yijk = \mu + Ti + Bj + Ck + eijk$; where;

Yijk = weekly body mass

μ = overall mean common to all observations

Ti = effect of treatment regimen (n = 1,2,..5)

Bj = fixed effect of individual rat (1,2,3.....98)

Ck = fixed effect of sampling week on body mass (= 1,2.....12)

eijk = residual random error

The model used for variables determined at study termination was:

$Yijk = \mu + Ti + Bj + eijk$; where;

Yijk = response variable of interest

μ = overall mean to all observations

Ti = effect of treatment (n = 1,2,..5)

Bj = fixed effect of individual rat (1,2,3.....98)

eijk = residual random error

CHAPTER 4: RESULTS

4.1 Phytochemical composition of the extract

Table 4.1 shows the total phenolics content and qualitative phytochemical composition of the hydroethanolic *O. ficus-indica* cladode extract.

The hydroethanolic *O. ficus-indica* cladode extract had a total phenolic content of 16 mg of gallic acid equivalent (GAE)/100g of dry extract and contained saponins and tannins but did not appear to contain alkaloid, flavonoids and terpenoids

Table 4.1: Qualitative phytochemical composition and total phenolic content of the hydroethanolic *Opuntia ficus-indica* cladode extract

Phytochemical	Results
Alkaloids	Absent
Flavonoids	Absent
Saponins	Present
Tannins	Present
Terpenoids	Absent
Total phenolic content (GAE/100g)	16mg

4.2 Growth performance

The induction and terminal body masses, as well as the empty carcass masses of the male rat pups, are shown in Figures 4.1 and 4.2, respectively. Across treatment regimens, the male rat pups grew significantly ($p < 0.0001$) over the experimental period (Figure 4.1). However, the induction and terminal body masses of the male rat pups across treatment regimens were similar (Figure 4.1). Male rat pups fed the high-fructose high-fat and fenofibrate (SRCB + FS + FeC) had significantly ($p < 0.0001$) lower empty carcass masses as compared that of rats given other treatments (Figure 4.2).

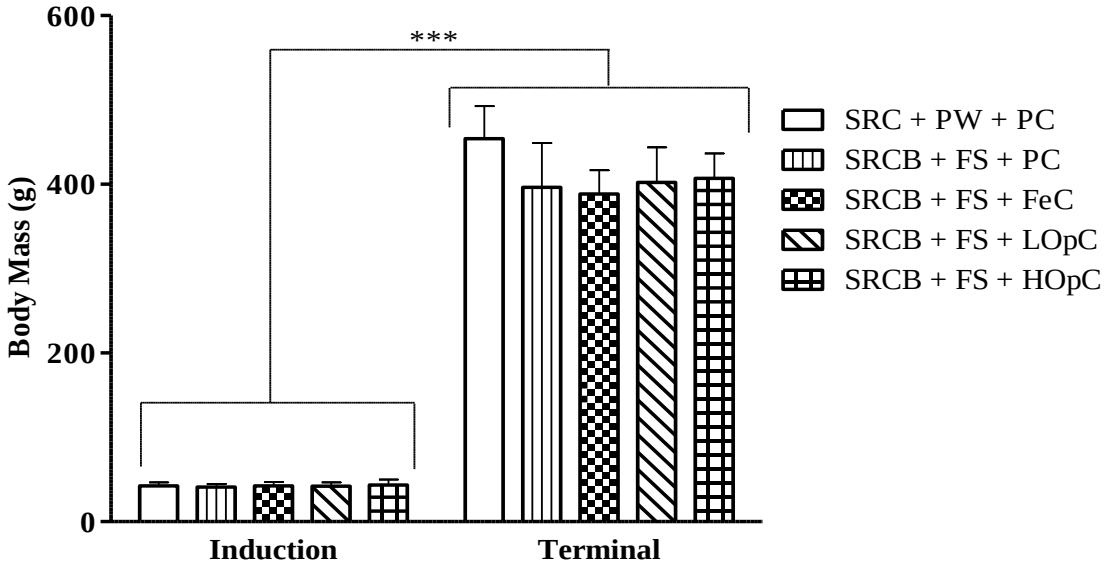

Figure 4.1: Induction and terminal masses of male rats fed high-fructose high-fat diet with hydroethanolic *Opuntia ficus-indica* cladode extract

*** $p < 0.0001$. The induction and terminal body masses of the male rats were similar. Across treatment regimens, the male rats significantly grew ($p < 0.0001$) compared to their induction masses. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

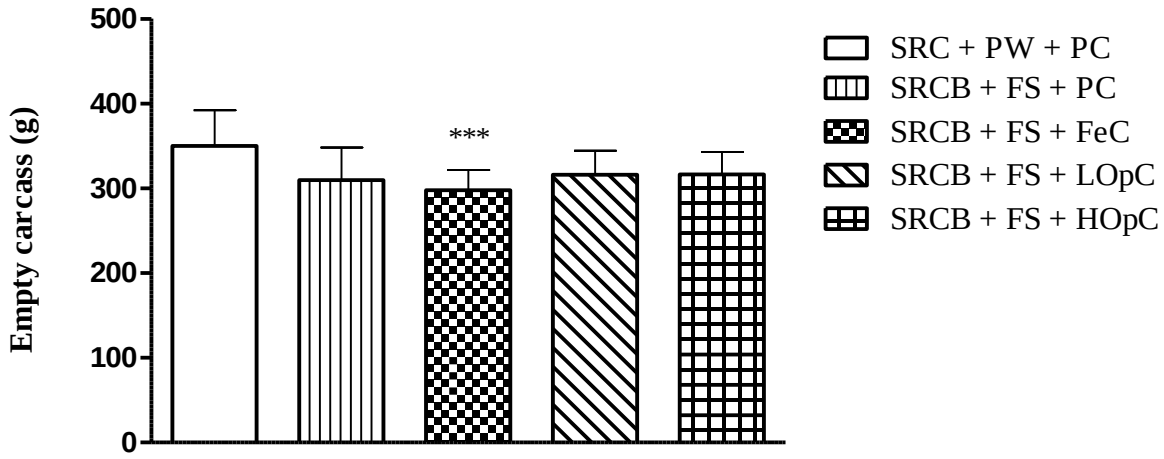

Figure 4.2: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on empty carcass mass of high-fructose high-fat diet-fed male rats

*** $p < 0.0001$. Rats given SRCB + FS + FeC had significantly ($p < 0.0001$) lower empty carcass mass as compared that of rats given other treatments. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

The induction and terminal body masses, and empty carcass masses of female rat pups are shown in Figure 4.3 and 4.4, respectively. Although the female rat pups across treatment regimens grew significantly ($p < 0.05$) over the experimental period (Figure 4.3), the induction and terminal body masses of the female rat pups across treatment regimens were similar (Figure 4.3). The female rats that were fed SRCB + FS + FeC had significantly ($p < 0.05$) lower empty carcass masses compared to that of rats given other treatments (Figure 4.4).

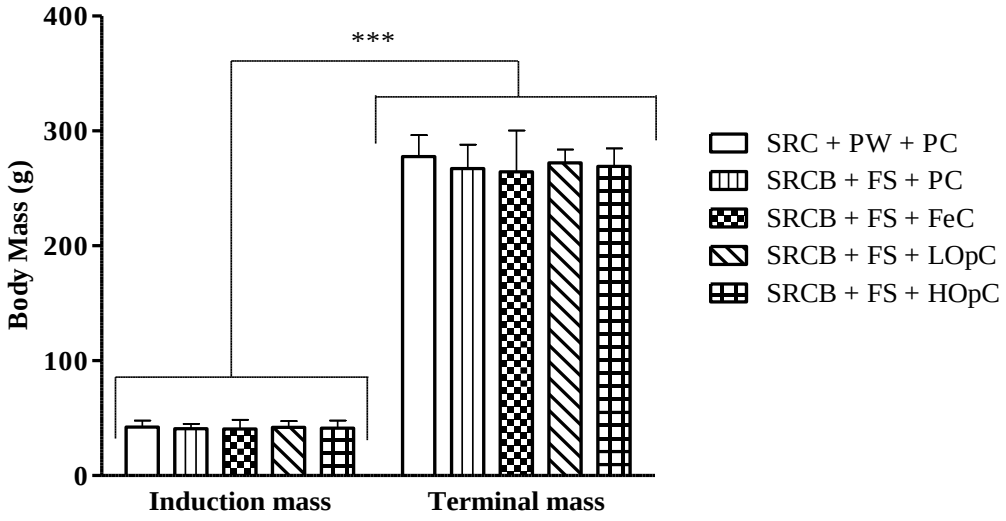

Figure 4.3: Induction and terminal masses of female rats fed high-fructose high-fat diet with hydroethanolic *Opuntia ficus-indica* cladode extract

*** $p < 0.0001$. The induction and terminal body masses of the rats were similar. Across treatment regimens, the rat pups grew significantly ($p < 0.0001$). SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

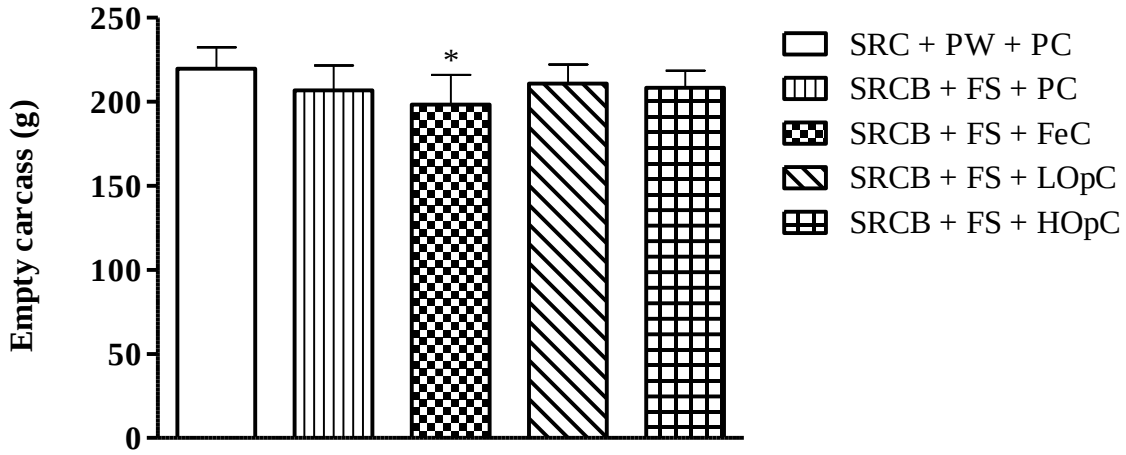

Figure 4.4: Effect of hydroethanolic *O. ficus-indica* cladode extract on empty carcass mass of high-fructose high-fat diet-fed female rats

* $p < 0.05$. Rats given SRCB + FS + FeC had a significantly ($p < 0.05$) lower empty carcass masses as compared to empty carcass masses of rats from all treatment groups. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

Tables 4.2 and 4.3 shows the tibia masses, lengths and mass to length ratio of male and female rats, respectively. In both male and female rats, treatment regimens had no effect on the tibiae masses, lengths, and mass to length ratio (Tables 4.2 and 4.3).

Table 4.2: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on the tibiae masses, lengths, and mass to length ratio of male rats fed a high-fructose high-fat diet

Tibia	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
Mass (mg)	667.5 ± 47.44	603.0 ± 55.48	615.6 ± 44.53	627.8 ± 68.32	602.3 ± 47.79	ns
Length (mm)	21.55 ± 0.72	21.06 ± 0.52	21.05 ± 0.53	21.58 ± 0.96	20.86 ± 0.57	ns
Mass to length ratio (mg/mm)	30.97 ± 1.74	28.67 ± 2.97	29.23 ± 1.77	29.05 ± 2.44	28.86 ± 1.91	ns

ns = not significant, $p > 0.05$. Tibiae lengths, masses and densities were similar across treatments. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

Table 4.3: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on the tibiae masses, lengths, and mass to length ratio of female rats fed a high-fructose high-fat diet

Tibia	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
Mass (mg)	533.7 ± 46.88	517.5 ± 52.32	485.2 ± 34.79	506.9 ± 43.04	496.9 ± 44.33	ns
Length (mm)	19.85 ± 0.57	19.58 ± 0.42	19.56 ± 0.34	19.74 ± 0.26	19.67 ± 0.95	ns
Mass to length ratio (mg/mm)	26.86 ± 1.91	26.41 ± 2.39	24.81 ± 1.78	25.69 ± 2.20	25.27 ± 2.08	ns

ns = not significant, $p > 0.05$. Tibiae lengths, masses and densities across all the treatment regimens were similar for rats. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

4.3 Tolerance to an oral glucose load

The blood glucose concentration at basal (0 min) and at 15, 30, 60 and 120 minutes post-gavage and area under the curve (AUC) calculated from OGTT of the male rats are illustrated below in Figure 4.5 and Figure 4.6, respectively. The rats given SRCB + FS + FeC had a significantly higher ($p < 0.0001$) blood glucose concentration compared to that of rats administered other treatments at all time intervals post gavage (Figure 4.5). While the blood glucose concentration of rats given SRC + PW + PC, SRCB + FS + FeC, SRCB + FS + LOpC and SRCB + FS + HOpC significantly peaked ($p < 0.0001$) at 15 mins post-gavage, the rats given SRCB + FS + PC significantly peaked ($p < 0.0001$) at 30 mins post gavage (Figure 4.6). Blood glucose concentrations of rats given rats given SRC + PW + PC, SRCB + FS + PC, SRCB + FS + LOpC and SRCB + FS + HOpC returned to baseline at 120 minutes post-gavage. Blood glucose concentrations of rats given SRCB + FS + FeC did not return to basal concentrations post-gavage at 120 minutes (Figure 4.5). The rats treated with SRCB + FS + FeC had significantly ($p < 0.05$) higher area under the curve of oral glucose tolerance test compared to that of rats from all other treatment (Figure 4.6).

The blood glucose concentration at basal (0 min) and at 15, 30, 60 and 120 minutes post-gavage and area under the curve (AUC) calculated from OGTT of the female rats are illustrated below in Figure 4.7 and Figure 4.8, respectively. The rats given SRCB + FS + FeC had a significantly higher ($p < 0.0001$) blood glucose concentration compared to all other treatment groups at all time intervals post gavage, except at 60 minutes post-gavage where they had significantly higher ($p = 0.0005$) blood glucose concentration compared to rats given SRC + PW + PC (Figure 4.7). The blood glucose concentration of rats given SRC + PW + PC, SRCB + FS + FeC and SRCB + FS + LOpC significantly peaked ($p < 0.0001$) at 15 mins post-gavage respectively, while that of rats given SRCB + FS + FeC and SRCB + FS + HOpC significantly peaked ($p < 0.0001$) at 30 mins post gavage. The blood glucose concentrations of rats from all treatments returned to basal concentrations at 120 mins post-gavage (Figure 4.7). Rats treated with SRCB + FS + FeC had significantly ($p < 0. 001$) higher area under the curve of oral glucose tolerance test compared to that of rats from all other treatment groups (Figure 4.8).

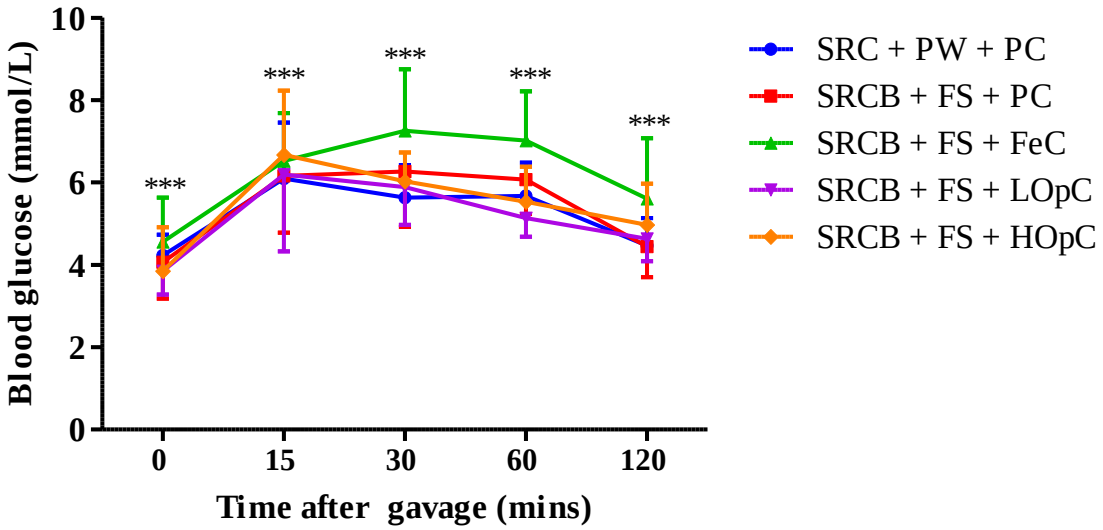

Figure 4. 5: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on the oral glucose tolerance of male rats fed a high-fructose high-fat diet

*** $p < 0.0001$. Rats given SRCB + FS + FeC had a significantly higher ($p < 0.0001$) blood glucose concentration compared to all the other treatment groups at all time intervals post gavage, except at 30 minutes post-gavage where they had significantly higher ($p = 0.0005$) blood glucose concentration compared to rats given SRC + PW + PC. The blood glucose concentration of rats given SRC + PW + PC, SRCB + FS + FeC, SRCB + FS + LOpC and SRCB + FS + HOpC significantly peaked ($p < 0.0001$) at 15 mins post-gavage, while that of rats given SRCB + FS + PC and significantly peaked ($p < 0.0001$) at 30 mins post gavage. Blood glucose concentration of rats given SRC + PW + PC, SRCB + FS + PC, SRCB + FS + LOpC and SRCB + FS + HOpC returned to baseline at 120 minutes post-gavage. The blood glucose concentration of rats given SRCB + FS + FeC did not return to basal concentration post-gavage. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

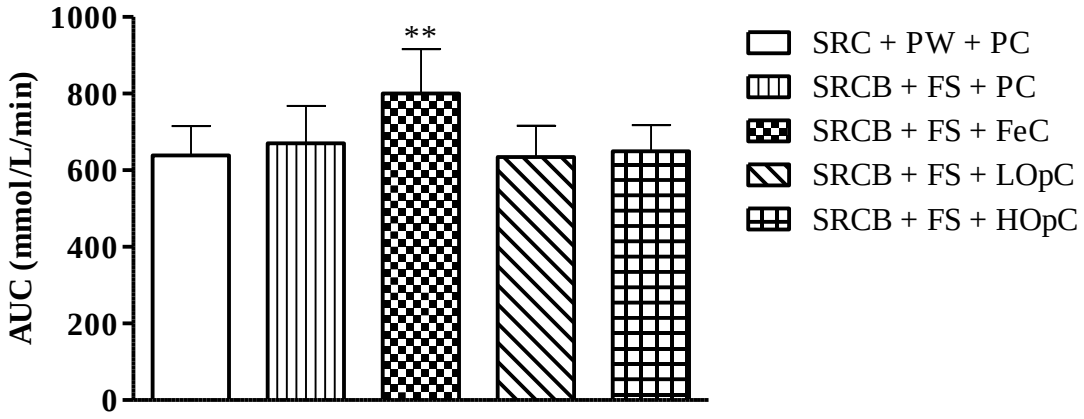

Figure 4. 6: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on total area under the curve of oral glucose tolerance of male rats fed a high-fructose high-fat diet

** p< 0.001. Rats fed SRCB + FS + FeC had significantly (p < 0.001) higher area under the curve of oral glucose tolerance test compared to that of rats from all other treatment groups. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

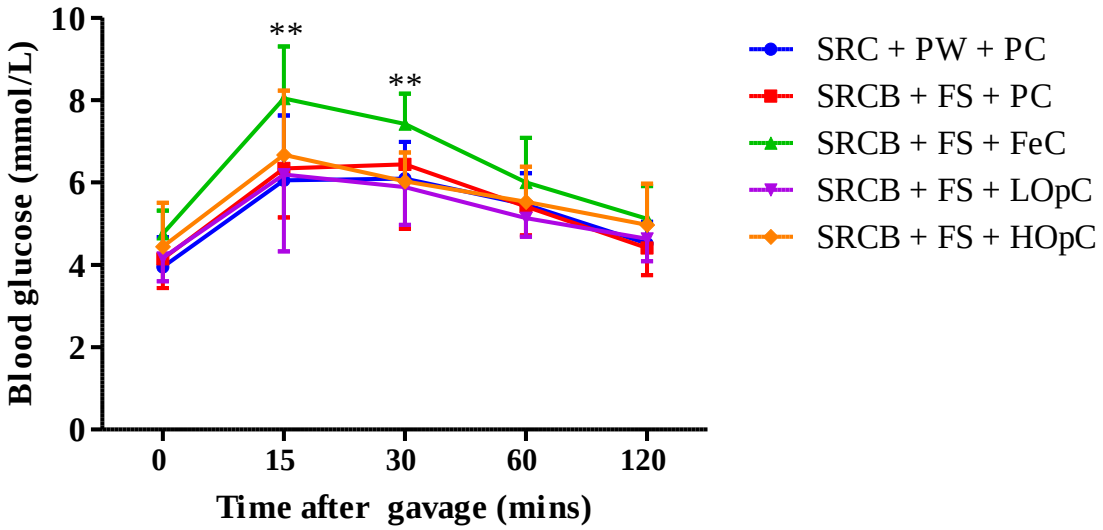

Figure 4. 7: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on the oral glucose tolerance of female rats fed a high-fructose high-fat diet

*** $p < 0.0001$. Rats given SRCB + FS + FeC had a significantly higher ($p < 0.0001$) blood glucose concentration compared to all the other treatment groups at all time intervals post gavage, except at 60 minutes post-gavage where they had significantly higher ($p = 0.0005$) blood glucose concentration compared to rats given SRC + PW + PC. The blood glucose concentration of rats given SRC + PW + PC, SRCB + FS + FeC and SRCB + FS + LOpC significantly peaked ($p < 0.0001$) at 15 mins post-gavage respectively, while that of rats given SRCB + FS + FeC and SRCB + FS + HOpC significantly peaked ($p < 0.0001$) at 30 mins post gavage. The blood glucose concentrations of rats from all treatment-regimens returned to basal concentrations at 120 mins post-gavage. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

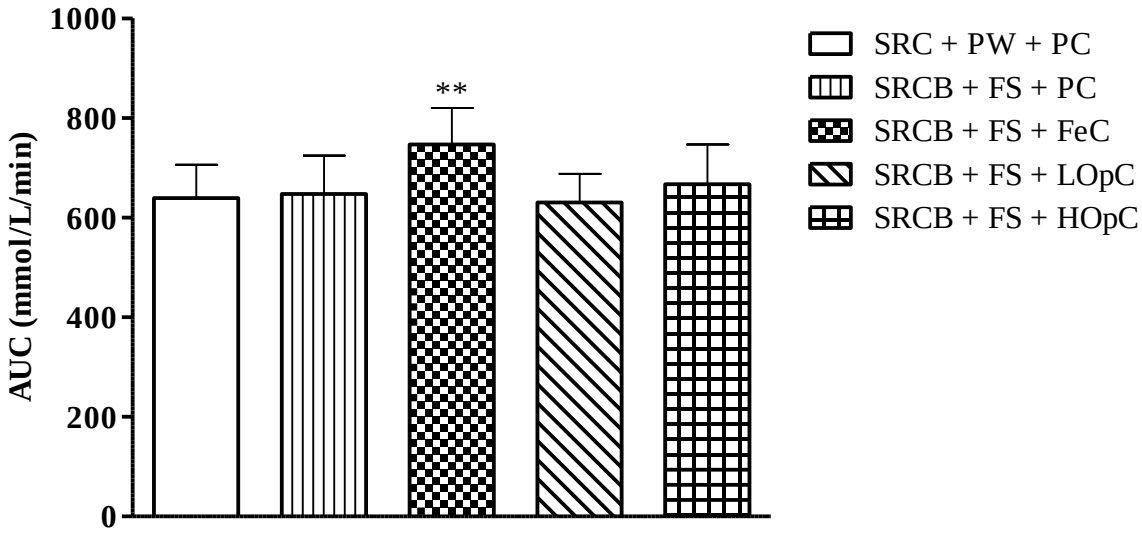

Figure 4. 8: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on total area under the curve of oral glucose tolerance of female rats fed a high-fructose high-fat diet

** $p < 0.001$. Rats treated with SRCB + FS + FeC had significantly ($p < 0.001$) higher area under the curve of oral glucose tolerance test compared to that of rats from all other treatment groups. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

4.4 Blood parameters and liver lipid content

The blood glucose, insulin, triglyceride, total cholesterol, HDL-cholesterol (HDL-chol) and TBARS concentration, as well as HOMA-IR of male rats, are shown in Table 4.4. The insulin, triglyceride, total cholesterol, HDL-cholesterol and TBARS concentration and HOMA-IR of the male rats were similar across treatment regimens. However, rats given SRCB + FS + FeC or SRCB + FS + HOpC had significantly ($p < 0.0011$) higher blood glucose concentration (Table 4.4).

Table 4.5 presents the blood glucose, insulin, triglyceride, total cholesterol, HDL-cholesterol (HDL-chol) and TBARS concentration as well as HOMA-IR of female rats. The insulin, triglyceride, total cholesterol, HDL-cholesterol and TBARS concentration and HOMA-IR of the female rats were similar across treatment regimens (Table 4.5).

Table 4.4: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on blood glucose, insulin, HOMA-IR, triglyceride, total cholesterol, HDL-cholesterol and TBARS of male rats fed a high-fructose high-fat diet

Parameter	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
Glucose (mmol/L)	3.57 ± 0.53[a]	3.75 ± 0.36[a]	4.41 ± 0.60[b]	3.50 ± 0.26[a]	4.06 ± 0.63[b]	**
Insulin (mmol/L)	1.67 ± 0.72[a]	1.79 ± 0.58[a]	1.71 ± 0.69[a]	1.47 ± 0.61[a]	1.75 ± 0.52[a]	ns
HOMA-IR	5.34 ± 2.79[a]	4.90 ± 1.54[a]	5.82 ± 1.91[a]	4.42 ± 2.00[a]	5.16 ± 1.67[a]	ns
TGs (mmol/L)	0.61 ± 0.12[a]	0.71 ± 0.19[a]	0.67 ± 0.20[a]	0.66 ± 0.28[a]	0.70 ± 0.19[a]	ns
T-chol (mmol/L)	0.89 ± 0.26[a]	0.76 ± 0.15[a]	0.83 ± 0.20[a]	0.78 ± 0.11[a]	0.75 ± 0.23[a]	ns
HDL-chol (mmol/L)	1.33 ± 0.21[a]	1.34 ± 0.22[a]	1.18 ± 0.33[a]	1.42 ± 0.33[a]	1.33 ± 0.31[a]	ns
TBARS (MDA)	0.01 ± 0.03[a]	0.03 ± 0.01 [a]	0.01 ± 0.04[a]	0.01 ± 0.03[a]	0.01 ± 0.01[a]	ns

ns = not significant, **p < 0.01. The insulin, HOMA-IR, triglyceride, total cholesterol, HDL-cholesterol and TBARS were similar in the rat pups across treatment regimens but the terminal glucose concentration was significantly (p < 0.0011) higher in rats given SRCB + FS + FeC and SRCB + FS + HOpC. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; HOMA-IR = Homeostatic Model Assessment of Insulin Resistance, TGs = triglycerides, T-chol = Total cholesterol, HDL-chol = HDL cholesterol, TBARS = Thiobarbituric acid reactive and MDA = malondialdehyde. Data presented as mean ± SD; n = 8 per treatment.

Table 4.5: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on blood glucose, insulin, HOMA-IR, triglyceride, total cholesterol, HDL-cholesterol and TBARS of female rats fed a high-fructose high-fat diet

Parameter	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
Glucose (mmol/L)	3.50 ± 0.45[a]	3.63 ± 0.47[a]	3.89 ± 0.33[a]	3.67 ± 0.46[a]	3.42 ± 0.38[a]	ns
Insulin (mmol/L)	1.08 ± 0.68[a]	1.14 ± 0.77[a]	1.65 ± 0.90[a]	1.30 ± 0.97[a]	1.29 ± 0.95[a]	ns
HOMA-IR	3.03 ± 2.02[a]	3.51 ± 2.47[a]	4.67 ± 2.57[a]	3.79 ± 2.99[a]	3.50 ± 2.57[a]	ns
TGs (mmol/L)	0.47 ± 0.21[a]	0.54 ± 0.18[a]	0.67 ± 0.34[a]	0.65 ± 0.31[a]	0.59 ± 0.25[a]	ns
T-chol (mmol/L)	0.93 ± 0.19[a]	1.01 ± 0.32[a]	1.01 ± 0.25[a]	1.09 ± 0.23[a]	0.94 ± 0.08[a]	ns
HDL-chol (mmol/L)	1.94 ± 0.33[a]	1.80 ± 0.45[a]	1.94 ± 0.34[a]	1.75 ± 0.34[a]	1.69 ± 0.36[a]	ns
TBARS (MDA)	0.004 ± 0.03[a]	0.013 ± 0.03[a]	0.008 ± 0.03[a]	0.009 ± 0.04[a]	0.004 ± 0.03[a]	ns

ns = not significant. The glucose, insulin, HOMA-IR, triglyceride, total cholesterol, HDL-cholesterol and TBARS were similar in the rat pups across treatment regimens. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; HOMA-IR = Homeostatic Model Assessment of Insulin Resistance, TGs = triglycerides, T-chol = Total cholesterol, HDL-chol = HDL cholesterol, TBARS = Thiobarbituric acid reactive and MDA = malondialdehyde. Data presented as mean ± SD; n = 8 per treatment.

The total hepatic lipid content of male rat pups following administration of their respective treatments is shown in Figure 4.9. In male rats, the consumption of the high-fructose high-fat diet significantly increased ($p < 0.0001$) liver lipid accretion (Figure 4.9). The low dose of the hydroethanolic *O. ficus-indica* cladode extract and fenofibrate prevented the increased liver lipid accretion in the high-fructose high-fat diet-fed male rats. However, the high dose of the hydroethanolic *O. ficus-indica* cladode extract only attenuated ($p < 0.0018$ vs SRCB + FS + PC) the high-fructose high-fat diet-induced increase in liver lipid accretion in male rats (Figure 4.9).

The total hepatic lipid content of female rat pups following administration of their respective treatments is shown in Figure 4.10. The female rats given SRCB + FS + PC had a significantly higher ($p < 0.0001$) liver lipid content compared to counterparts administered the SRCB + FS + FeC, SRCB + FS + LOpC and SRCB + FS + HOpC (Figure 4.10). In female rats, the low dose hydroethanolic *O. ficus-indica* cladode extract attenuated the high-fructose high-fat diet diet-induced increase in lipid accretion (Figure 4.10). However, the high dose hydroethanolic *O. ficus-indica* cladode extract (SRCB + FS + HOpC) attenuated the high-fructose high-fat diet diet-induced increase in lipid accretion (Figure 4.10).

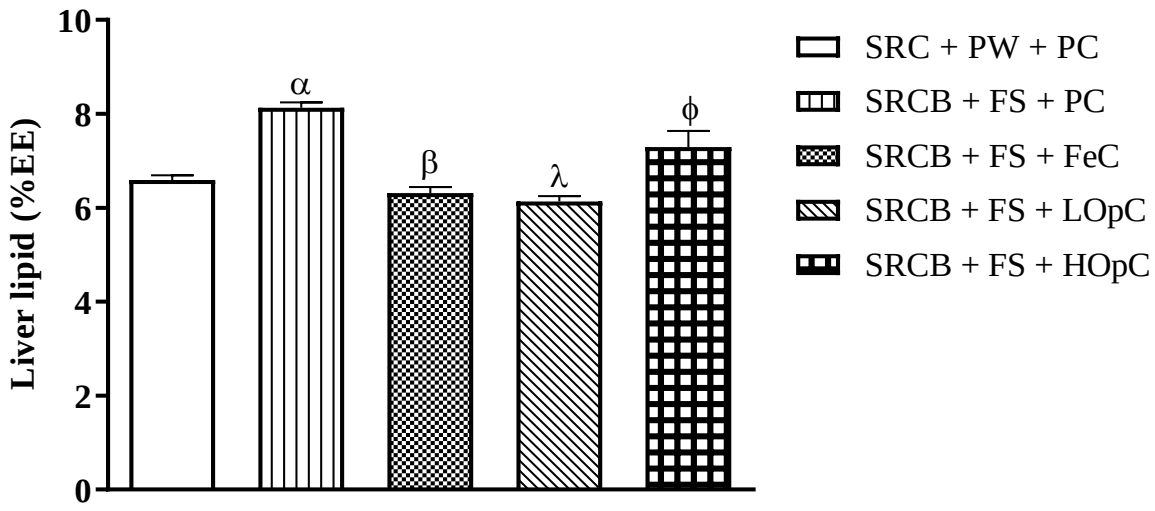

Figure 4. 9: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on the total hepatic lipid content of male rats fed a high-fructose high-fat diet

$^{\alpha}$ $p < 0.0001$ compared to all the other treatment regimens. $^{\beta}$ $p < 0.05$ compared to SRCB + FS + PC and SRCB + FS + HOpC, $^{\lambda}$ $p < 0.0001$ compared to all other treatment regimens. $^{\phi}$ $p < 0.0001$ when SRCB + FS + HOpC was compared to the other treatment regimens. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; EE = ether extract. Data presented as mean ± SD; n = 9-10 per treatment.

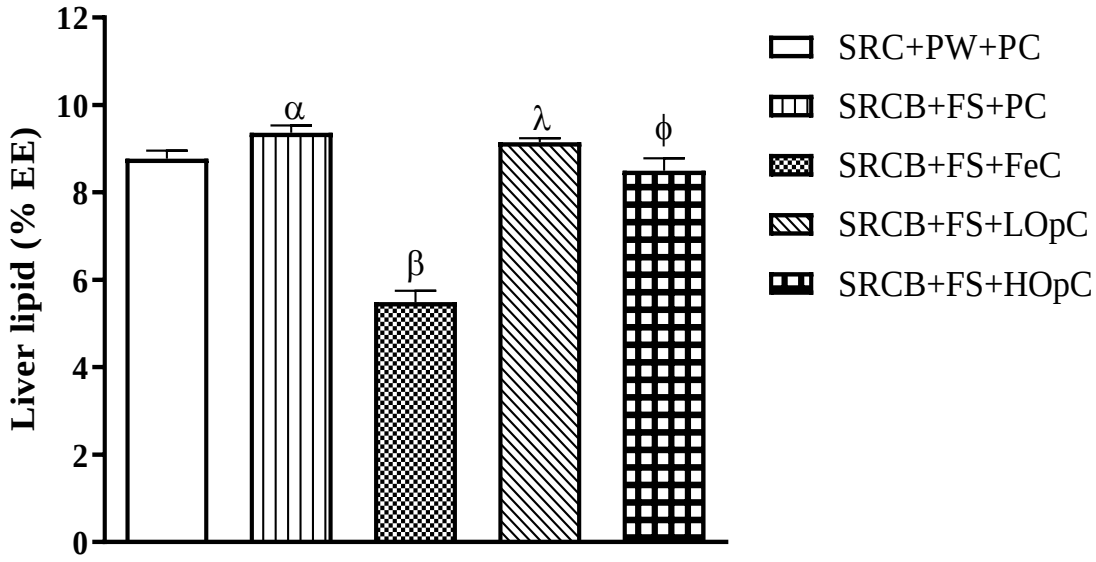

Figure 4. 10: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on the total hepatic lipid content of female rats fed a high-fructose high-fat diet

$^{\alpha}$ $p < 0.0001$ when SRCB + FS + PC was compared to SRCB + FS + FeC, SRCB + FS + LOpC and SRCB + FS + HOpC, $^{\beta}$ $p < 0.0001$ when SRCB + FS + FeC was compared to all the other treatment regimens. $^{\lambda}$ $p < 0.05$ when SRCB + FS + LOpC was compared to all other treatment regimens. $^{\phi}$ $p < 0.05$ when SRCB + FS + HOpC was compared to all the other treatment regimens. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. Data presented as mean ± SD; n = 9-10 per treatment.

4.5 Viscera macro-morphometry

Table 4.6 below shows male rat pups' absolute and relative (to body mass) liver, kidney, epididymal and visceral fat, absolute masses. The livers of male rats given SRCB + FS + FeC were significantly heavier ($p < 0.0001$) compared to those of the rats in other treatment regimens. However, their visceral fat and epididymal fat mass (absolute and relative) were significantly lower ($p < 0.05$) compared to those of the rats in other treatment regimens (Table 4.6). The relative (to body mass) kidneys, visceral fat and epididymal fat masses of male rats given SRCB + FS + PC and SRCB + FS + FeC were significantly heavier ($p< 0.0005$) compared to that of rats given SRCB + FS + LOpC and SRCB + FS + HOpC (Table 4.6). The rest of male rat pups' viscera masses (absolute and relative) and lengths were similar across treatments (Table 4.6).

Table 4.7 below shows female rat pups' liver, kidney and visceral fat, absolute and relative (to body mass) masses. Though the absolute visceral fat masses of female rats given SRCB + FS + FeC were significantly lower ($p < 0.05$) compared to that from their counterparts given SRCB + FS + LOpC and SRCB + FS + HOpC, their liver masses (absolute and relative) were significantly higher ($p < 0.0001$) compared to female rats that received other treatments. The absolute and relative mass of the kidney was the same (Table 4.7) throughout the treatments.

4.6 Liver histo-morphometry

Representative liver histo-morphometry photo sections (H and E staining, 400 X magnification) of male rat pups from the different treatment regimens are presented in Figure 4.11 below. Steatosis, lobular inflammation and ballooning were not observed across treatment regimens (Figure 4.11).

Representative liver histo-morphometry photo sections (H and E staining, 400 X magnification) of female rat pups from the different treatment regimens are presented in Figure 4.12 below. Steatosis, lobular inflammation and ballooning were not observed across treatment regimens (Figure 4.12).

Table 4.6: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on liver, kidney, epididymal fat and visceral fat masses of high-fructose high-fat diet-fed male rat pups

Organ	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
Liver (g)	12.11 ± 0.92^{a}	11.42 ± 1.01^{a}	15.99 ± 1.69^{b}	11.46 ± 1.25^{a}	11.43 ± 1.19^{a}	***
Liver (%BM)	2.67 ± 0.10^{a}	2.91 ± 0.32^{a}	4.12 ± 0.36^{b}	2.85 ± 0.2^{a}	2.81 ± 0.2^{a}	***
Kidneys (g)	2.81 ± 0.22^{a}	2.53 ± 0.21^{a}	2.73 ± 0.21^{a}	2.48 ± 0.21^{a}	2.52 ± 0.25^{a}	ns
Kidneys (%BM)	0.58 ± 0.07^{a}	0.65 ± 0.09^{b}	0.70 ± 0.03^{ab}	0.62 ± 0.04^{b}	0.62 ± 0.05^{b}	**
Visceral fat (g)	11.28 ± 1.93^{a}	9.74 ± 2.17^{b}	7.30 ± 2.71^{b}	9.32 ± 2.40^{ab}	10.59 ± 1.96^{a}	**
Visceral fat (%BM)	2.71 ± 0.50^{a}	2.56 ± 0.29^{b}	2.05 ± 0.33^{b}	2.30 ± 0.53^{ab}	2.70 ± 0.32^{a}	**
Epididymal fat (g)	4.14 ± 0.88^{a}	3.41 ± 0.96^{b}	2.47 ± 0.32^{b}	3.19 ± 0.78^{ab}	3.30 ± 0.97^{ab}	*
Epididymal fat (%BM)	0.91 ± 0.15^{a}	0.79 ± 0.21^{b}	0.64 ± 0.09^{b}	0.79 ± 0.17^{ab}	0.81 ± 0.02^{a}	*

ns = not significant, $p > 0.05$; * $p < 0.05$; **$p < 0.01$; *** $p < 0.0001$. ^{ab}Within row means with different superscripts are significantly different at $p < 0.05$. Rats given SRCB + FS + PC and SRCB + FS + FeC had significantly heavier ($p < 0.0001$) absolute and relative (to body mass) liver, and significantly lower ($p < 0.05$) visceral fat and epididymal fat masses compared to rats given the other treatments. Rats given SRCB + FS + PC and SRCB + FS + FeC had significantly heavier ($p < 0.0005$) relative (to body mass) kidneys, visceral fat and epididymal fat masses compared to rats given SRCB + FS + LOpC and SRCB + FS + HOpC. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. BM = Body mass. Data presented as mean ± SD; n = 9-10 per treatment.

Table 4.7: Effect of hydroethanolic *Opuntia ficus-indica* cladode extracts on liver, kidney and visceral fat masses high-fructose high-fat diet-fed female rats

Organ	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
Liver (g)	7.51 ± 0.54[a]	7.72 ± 0.91[a]	10.15 ± 1.41[b]	7.85 ± 0.67[a]	7.56 ± 0.45[a]	***
Liver (%BM)	2.65 ± 0.15[a]	2.83 ± 0.21[a]	3.79 ± 0.16[b]	2.88 ± 0.19[a]	2.81 ± 0.15[a]	***
Kidneys (g)	1.82 ± 0.10[a]	1.71 ± 0.15[a]	1.79 ± 0.23[a]	1.75 ± 0.10[a]	1.70 ± 0.16[a]	ns
Kidneys (%BM)	0.66 ± 0.05[a]	0.64 ± 0.04[a]	0.68 ± 0.02[a]	0.64 ± 0.03[a]	0.63 ± 0.04[a]	ns
Visceral fat (g)	12.35 ± 1.46[a]	11.72 ±3.41[a]	8.56 ± 3.01[b]	11.80 ± 2.50[a]	11.15 ± 3.63[a]	ns
Visceral fat (%BM)	4.47 ± 0.58[a]	4.34 ± 0.99[a]	3.46 ± 0.90[a]	4.50 ± 0.65[a]	3.95 ± 1.16[a]	ns

ns = not significant, $p > 0.05$. * $p < 0.05$ *** $p < 0.0001$. [ab]Within row means with different superscripts are significantly different at $p \leq 0.05$. Rats given SRCB + FS + FeC had significantly lower ($p < 0.05$) absolute visceral fat masses and significantly heavier ($p < 0.0001$) livers (absolute and relative) compared to female rats that received other treatments. The absolute and relative (body mass) of the kidney was the same across the treatment regimens. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. BM = Body mass. Data presented as mean ± SD; n = 9-10 per treatment.

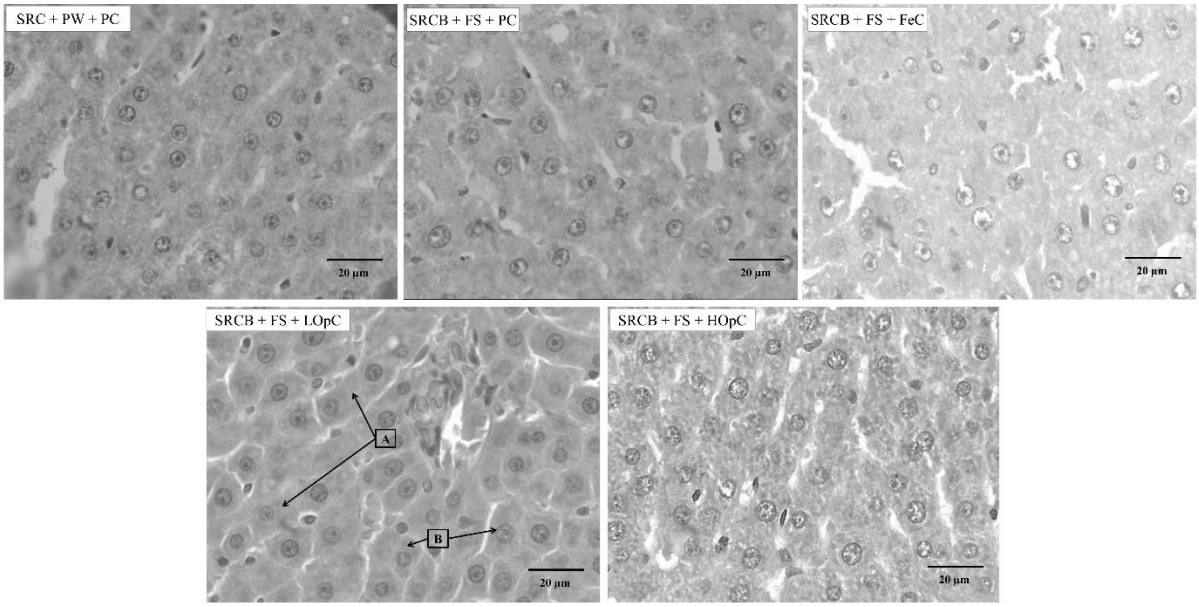

Figure 4. 11: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on pictures of liver histology (H and E staining, 400 X magnification) of male rats fed a high-fructose high-fat diet

Arrows A indicates hepatocytes; arrows B indicate sinusoids. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract.

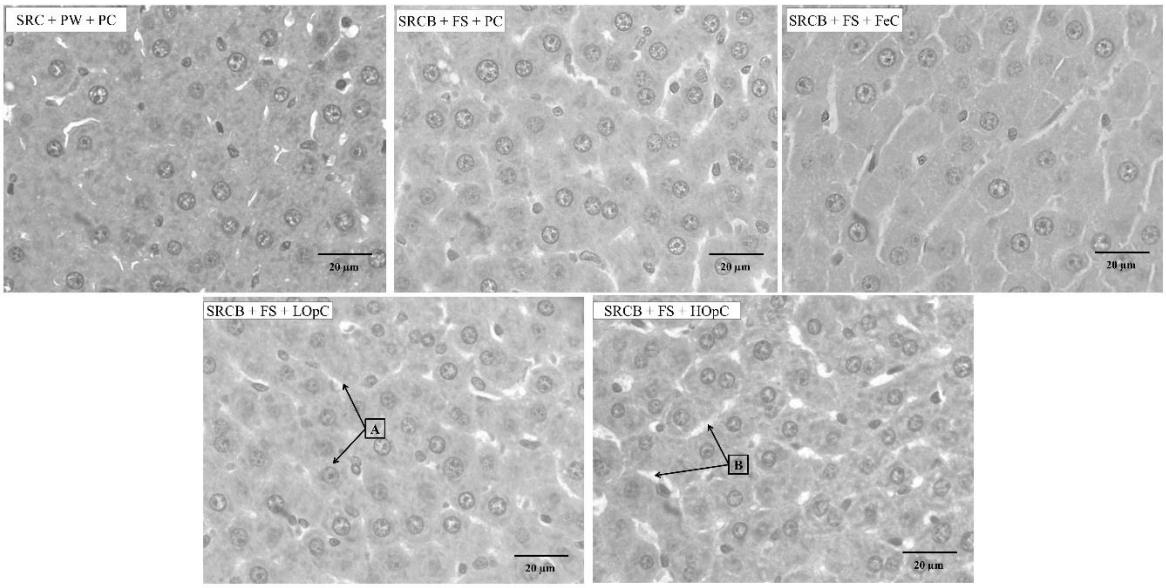

Figure 4. 12: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on pictures of liver histology (H and E staining, 400 X magnification) of female rats fed a high-fructose high-fat diet

Arrows A indicates hepatocytes; arrows B indicate sinusoids. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract.

4.7 Plasma surrogate markers of liver and kidney function

Table 4.8 shows the plasma activities of aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) and plasma creatinine and blood urea nitrogen (BUN) concentrations of the male rats. The plasma ALP activity of male rats given SRCB + FW + FeC were significantly higher ($p < 0.0001$) compared to that of male rats given SRCB + FS + PC, SRC + PW + PC, SRCB + FS + LOpC and SRCB + FS + HOpC (Table 4.8). However, the plasma activities of AST and GGT, as well as plasma BUN and creatinine concentrations of male rats, were similar across treatments (Table 4.8).

Table 4.9 shows the plasma activities of AST, ALP and GGT as well as plasma creatinine and blood urea nitrogen (BUN) concentrations of the female rats. The plasma surrogate markers of liver and kidney function of female rats were similar across treatment regimens (Table 4.9).

Table 4. 8: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on plasma surrogate markers of high-fructose high-fat diet-fed male rats

Parameter	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
AST (U/L)	96.33 ± 28.52[a]	88.0 ± 18.00[a]	89.75 ± 12.97[a]	86.29 ± 11.15[a]	90.80 ± 32.45[a]	ns
ALP (U/L)	156.0 ± 27.22[a]	156.3 ± 18.44[a]	232.0 ± 54.73[b]	139.7 ± 26.61[ab]	165.0 ± 47.78[ab]	***
GGT (U/L)	nd	nd	nd	nd	nd	ns
BUN (mg/dL)	84.29 ± 18.84[a]	87.14 ± 20.18[a]	88.57 ± 16.76[a]	75.86 ± 16.19[a]	69.67 ± 16.19[a]	ns
Creatinine (mg/dL)	21.47 ± 4.73[a]	18.94 ± 3.34[a]	18.96 ± 3.33[a]	20.21 ± 4.31[a]	20.21 ± 6.68[a]	ns

ns = not significant, $p < 0.0001$. [ab] Within row means with different superscripts are significantly different at $p \leq 0.05$. Rats given SRCB + FS + FeC had significantly higher ($p < 0.0001$) alkaline phosphatase concentration compared to rats given SRCB + FS + PC and SRC + PW + PC. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. AST = aspartate amino transferase, ALP = alkaline phosphatase, GGT = gamma-glutamyl transferase and BUN = blood urea nitrogen, nd = not detected. Data presented as mean ± SD; n = 7 per treatment.

Table 4. 9: Effect of hydroethanolic *Opuntia ficus-indica* cladode extract on plasma surrogate markers of high-fructose high-fat diet-fed female rats

Parameters	SRC + PW + PC	SRCB + FS + PC	SRCB + FS + FeC	SRCB + FS + LOpC	SRCB + FS + HOpC	Significance
AST (U/L)	117.4 ± 30.82[a]	104.5 ± 33.49[a]	93.14 ± 31.46[a]	107.3 ± 64.65[a]	94.71 ± 27.75[a]	ns
ALP (U/L)	121.0 ± 19.62[a]	124.8 ± 47.55[a]	144.0 ± 32.53[a]	107.6 ± 27.15[a]	126.9 ± 21.43[a]	ns
GGT (U/L)	nd	nd	nd	nd	nd	ns
BUN (mg/dL)	75.29 ± 20.34[a]	74.33 ± 19.83[a]	67.86 ± 14.21[a]	65.71 ± 15.49[a]	76.00 ± 15.29[a]	ns
Creatinine (mg/dL)	22.73 ± 4.73[a]	20.63 ± 4.57[a]	22.73 ± 4.73[a]	21.47 ± 4.73[a]	20.21 ± 4.31[a]	ns

ns = not significant, $p > 0.05$. The plasma surrogate markers were similar across all treatment regimens. SRC + PW + PC = standard rat chow + plain drinking water + plain gelatine cube; SRCB + FS + PC = standard rat chow with 2% w/w beef tallow + 25 % w/v fructose solution + plain gelatine cube; SRCB + FS + FeC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 100 mg/kg body mass fenofibrate; SRCB + FS + LOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 50 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract; SRCB + FS + HOpC = standard rat chow with 2% w/w beef tallow + 25% w/v fructose solution + 500 mg/kg body mass of hydroethanolic *O. ficus-indica* cladode extract. AST= aspartate amino transferase, ALP = alkaline phosphatase, GGT = gamma-glutamyl transferase and BUN = blood urea nitrogen, nd = not detected. Data presented as mean ± SD; n = 7 per treatment.

CHAPTER 5: DISCUSSION

5.0 Discussion

The prevalence of obesity, NAFLD, MetS and T-II-DM in children is on the rise. Animal and human studies have shown that the consumption of diets that have high fructose and fat content results in obesity, MetS and NAFLD (Saklayen 2018). Thus, there is a need for studies to find plant-derived ethnomedicines that can be used to prevent diet-induced metabolic disorders. Therefore, the current study aimed to determine the phytochemical composition qualitatively and the phenolic content quantitatively of hydroethanolic *O. ficus-indica* cladode extract and whether the extract can protect against high-fructose high-fat diet-induced metabolic derangements in growing rats modelling children fed obesogenic diets.

5.1 Phytochemical composition and total phenolic content

Total phenolic content is used as an indicator of the total amount of bioactive phytochemical compounds (Aryal et al. 2019). The total phenolic content of ethnomedicinal extracts can be influenced by the climate of the region where the plant was obtained as well as by the extraction method (Bensadón et al. 2010). Studies have reported that the total phenolic content in commercial and wild *O. ficus-indica* cladode extracts from European and African countries ranges from 1.24 to 5.41mg gallic acid equivalent (GAE)/100g (Alves et al. 2017; Haile et al. 2016). The current study reports that the hydroethanolic *O. ficus-indica* cladode extract had a higher total phenolic content of 16mg GAE/100g. The variance in the total phenolic content of *O. ficus-indica* cladode extract from the current study and those reported by previous studies (Haile et al. 2016; Alves et al. 2017; Rocchetti et al. 2018) could be attributed to the differences in the climate of the regions where the *O. ficus-indica* cladodes were obtained as well as extraction methods. Previous studies have reported that *O. ficus-indica* cladodes contain saponins, alkaloids, flavonoids, tannins and terpenoids which exert an array of health beneficial effects (Allai et al. 2016; El-Mostafa et al. 2014; Souza 2014). In the current study, based on the findings from the qualitative phytochemical tests, the hydroethanolic *O. ficus-indica* cladode extract was found to contain saponins and tannins but did not contain alkaloids, flavonoids and terpenoids. The presence of tannins and saponins suggests that my hydroethanolic *O. ficus-indica* cladode extract may possess antioxidant, antidiabetic and anti-obesogenic effects.

5.2 Hydroethanolic *Opuntia ficus-indica* cladode extracts in high-fructose high-fat diet-fed rats

The consumption of high-fructose high-fat diets has been reported to increase body mass (Pereira et al. 2017). In the current study, the consumption of a high-fructose high-fat diet had no effect on body mass gain of the male and female rats (Figures 4.1 and 4.3, respectively). The current study also reports that the hydroethanolic *O. ficus-indica* cladode extract and fenofibrate had no effect on body mass (Figure 4.3). These findings suggest that the hydroethanolic *O. ficus-indica* cladode extract and fenofibrate can be used without adversely affecting body mass gain.

Body mass is an inadequate anthropometric technique (Bower et al. 2017) since in the short-term, it can be influenced by gut-fill, viscera organ mass and hydration status (Mörgeli et al. 2017). These limitations make body mass an inaccurate index for assessing body composition and or growth (Llewellyn et al. 2016). Empty (eviscerated) carcass mass is used as an index of lean body mass (Mörgeli et al. 2017). In the current study, the high-fructose high-fat diet and hydroethanolic *O. ficus-indica* cladode extract had no effect on empty carcass mass (Figure 4.2). On the other hand, fenofibrate reduced the empty carcass masses of male and female rats fed high-fructose high-fat diet in the present study (Figure 4.2 and 4.4). These findings suggest that fenofibrate might reduce lean mass through increased beta-oxidation when used by children. Our findings are in agreement with Carmona et al. (2005) and Rivera-Meza et al. (2017), who reported that fenofibrate reduces lean body mass in rat models of diet-induced obesity through its effect on tissue lipid metabolism. Importantly, our findings suggest that, unlike fenofibrate (positive control), the hydroethanolic *O. ficus-indica* cladode extract can be administered to children for medicinal purposes without compromising the accretion of lean mass.

Consumption of high-fructose high-fat diet is known to increase liver lipid synthesis and accumulation as well as cause the development of NAFLD (Kwon *et al.*, 2016). The excess hepatic lipids that result from high-fructose high-fat diet intake are transported to visceral adipose tissue where they are stored and cause central obesity (Rafieian-Kopaei et al. 2014). Central (visceral) obesity is associated with increased oxidative stress, impaired glycaemic control, insulin resistance and dyslipidaemia (Lamming et al. 2013). Glycaemic control refers to the ability to regulate and maintain blood glucose concentrations within the homeostatic range (Lamming et al. 2013). The oral glucose tolerance test and area under the curve of oral

glucose tolerance test are used to evaluate glycaemic control (Zhang et al. 2013). In the current study, the high-fructose high-fat diet and the hydroethanolic *O. ficus-indica* cladode extract had no effect on glycaemic control (Figure 4.5 and 4.7). These findings are at variance with other studies that have shown that the consumption of high-fructose high-fat diet results in hyperglycaemia and impaired glycaemic control (Akash *et al.*, 2013; Healy *et al.*, 2015; Aragona *et al.*, 2018). Interestingly, the current study found that fenofibrate resulted in hyperglycaemia and impaired glycaemic control in growing male and female rats fed a high-fructose high-fat diet (Figures 4.5, 4.6, 4.7 and 4.8). These findings are consistent with Lui et al. (2011) and Kostapanos et al. (2013) who reported that fenofibrate impairs glycaemic control by increasing pro-inflammatory mediators that impaired insulin synthesis and secretion and induced insulin resistance. Thus, the use of fenofibrate should be used with caution as it could conceivably impair glycaemic control.

Diets that are rich in fructose and fat are known to increase lipogenesis as well as inhibit fatty acid oxidation (de Sousa Rodrigues et al. 2017; Sears and Perry 2015). The increased lipogenesis can cause hepatic steatosis, dyslipidaemia, visceral obesity, insulin resistance and increased oxidative stress (Ipsen *et al.*, 2018). The current study reports that the high-fructose high-fat diet feeding resulted in increased liver lipid accretion in male and female rats (Figure 4.9 and 4.10). At a low dose, my hydroethanolic *O. ficus-indica* cladode extract prevented the high-fructose high-fat diet-induced liver lipid accretion in male and female rats (Figure 4.9). Interestingly, the high dose of hydroethanolic *O. ficus-indica* cladode extract attenuated the development of high-fructose high-fat diet-induced liver lipid accretion in male and female rats (Figures 4.9 and 4.10, respectively). Fenofibrate prevented the high-fructose high-fat diet-induced excessive hepatic lipid accretion in male and female rats (Figure 4.9 and 4.10). Several studies have reported that fenofibrate prevents diet-induced liver lipid accretion by stimulating hepatic fatty oxidation (Carmona et al. 2005; Rivera-Meza et al. 2017). Thus, it is likely that in the current study, fenofibrate prevented the liver lipid high-fructose high-fat diet-induced liver lipid accretion in female rats by stimulating hepatic fatty oxidation. The *O. ficus-indica* cladode extract used in the current study contained tannins and saponins that are known to mitigate diet-induced fatty-liver disease by suppressing mediators of inflammation and preventing hepatic lipid deposition (Tchernof and Despres, 2013; Osuna-Martínez *et al.*, 2014; Taher *et al.*, 2016). It could be speculated that in the current study the hydroethanolic *O. ficus-indica* cladode extract attenuated the high-fructose high-fat diet-induced liver lipid accretion in the male and female rats by suppressing mediators of hepatic lipid accretion.

Excess lipolysis results in high free fatty acid (FFA) flux into the liver, where FFAs cause steatosis and exert a lipotoxic effect which leads to an increased hepatic lipid accretion. Although, the high-fructose high-fat diet mediated increased liver lipid accretion it did not cause steatosis, lobular inflammation and hepatocyte ballooning (Figure 4.11 and 4.12). According to literature the formation of steatosis occurs when hepatic lipid accretion is ≥ 10% of the dry liver mass (Nassir et al. 2015; Ipsen et al. 2018). This could explain the absence of steatosis despite an increase in liver lipid accretion in the high-fructose high-fat diet-fed rats.

Liver mass can be influenced by metabolic substrates content, hepatic inflammation and hepatomegaly (Senaphan et al. 2015). Despite the high-fructose high-fat diet causing an increase in liver lipid accretion in the rats, it had no effect on their liver masses in the current study. On the other hand, fenofibrate increased the liver mass of the rats (Table 4.6 and 4.7). Previous studies have shown that fenofibrate increases the liver mass (Kopf et al. 2014; Yan et al. 2014). The mechanism by which fenofibrate cause hepatomegaly is not fully understood, however, it is speculated that PPARα activation stimulates hepatic tissue proliferation (Michalik *et al.*, 2004). Therefore, the use of fenofibrate may cause hepatomegaly and thus liver health should be monitored closely when using fenofibrate. Unlike fenofibrate, the hydroethanolic *O. ficus-indica* cladode extract did not affect the liver mass of the rats (Table 4.6 and 4.7) suggesting that its use as an ethnomedicine is unlikely to result in hepatomegaly.

Aspartate aminotransferase (AST) is a transaminase enzyme that catalyses the conversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate (Aragona et al. 2018). Clinically an elevation of plasma AST activity indicates intrahepatic damage (Van Herck et al., 2017). Alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) are cholestatic enzymes (Wu *et al.*, 2016), that are found mainly in cells of the biliary tract. Thus, elevated plasma ALP and GGT activities indicate obstruction and or damage in the biliary tract (Subramaniam et al. 2015; Kwon et al. 2016). However, an increase in plasma ALP activity can also occur as a result of increased bone ossification (Subramaniam *et al.*, 2015). On the other hand, elevated GGT plasma activity can also signify alcohol-induced intrahepatic damage (Kwon et al. 2016; Wu et al. 2016). In my study, the fenofibrate resulted in elevated plasma ALP activity only in the male rats (Table 4.8) but did not affect GGT (Table 4.8 and 4.9). These findings suggest that the fenofibrate-induced increase in plasma ALP activity was not due to damage in the biliary tract. It is possible that the observed

increase in plasma ALP activity may have been due to activities occurring in the bones. Nonetheless, the underlying mechanisms pertaining to this finding are unclear and thus require further research. Fenofibrate had no effect on plasma AST activity of the rats, thus suggesting that despite it causing hepatomegaly, it did not cause hepatocyte damage.

The high-fructose high-fat diet and the hydroethanolic *O. ficus-indica* cladode extract have no effect on the liver function (Table 4.8 and 4.9). Thus, the use of hydroethanolic *O. ficus-indica* cladode extract as an ethnomedicine is unlikely to compromise liver function. However, I found that the high-fructose high-fat diet increased liver lipid content but did not cause hepatic steatosis, inflammation and ballooning or damage. Therefore, I did not expect any increase in plasma AST activity since my findings suggested that the fatty-liver disease was probably in the early stages of development before vacuolation had begun to form.

Studies have reported that rats fed high-fructose high-fat diets develop of metabolic disorders that closely mimic the pathophysiology that occurs in humans (Sekar et al. 2017; Francisqueti et al. 2017). Moreover, high-fructose high-fat diets have been shown to increase deposition of fat in tissues and in oxidative stress and consequently cause the onset of obesity, MetS and NAFLD (Moreno-Fernández et al. 2018). In my study feeding a high-fructose high-fat diet had no effect on the visceral adiposity of the rats (Table 4.7). Visceral obesity results in a mild pro-inflammatory state which is associated with insulin resistance, hyperglycaemia and dyslipidaemia (Uranga and Keller 2019; Lopes et al. 2016). In this study, fenofibrate reduced visceral fat pad masses in high-fructose high-fat diet-fed male rats but not in female rats (Table 4.6). Fenofibrate has been reported to reduce visceral obesity by stimulating fatty acid oxidation via activation of PPARα receptors (Rigano et al. 2017; Dias et al. 2018). Although the hydroethanolic *O. ficus-indica* cladode extract used in this study contained saponins and tannins that are reported to possess activities that mediate fatty acid oxidation, it had no effect on the visceral fat pad masses of the rats.

Epididymal fat provides the gonads with energy during spermatogenesis (Chu et al. 2010). A decrease or increase above normal in epididymal fat accumulation is associated with infertility (El Salam 2018; Chu et al. 2010). In my study, the high-fructose high-fat diet and hydroethanolic *O. ficus-indica* cladode extract had no effect on the epididymal fat masses of the male rats. However, fenofibrate reduced epididymal fat in high-fructose high-fat diet-fed rats (Table 4.6), suggesting that it may affect fertility in male rats. Nonetheless, this finding requires further interrogation.

In the current study, the high-fructose high-fat diet, hydroethanolic *O. ficus-indica* cladode extract and fenofibrate had no effect on blood triglyceride and cholesterol concentrations, insulin resistance, and peroxidation of the male and female rats (Figure 4.5 and 4.7). Previous studies have reported that high-fructose high-fat diet causes MetS (Moreno-Fernández et al. 2018; Jeong and Kim 2019). The difference in my findings and previous studies may be attributed to the young age of the rats that were used in this study. Lai et al., (2014) reported at a young age, rats are more resistant to developing robust metabolic disorders.

An increase in kidney mass is associated with kidney damage (Leveridge and Jewett 2011; Motzer et al. 2019). Previous studies have reported the consumption of a high-fructose high-fat diet can cause renal damage through the systemic release of pro-inflammatory mediators (uric acid and C-reactive protein) and through oxidative stress (Bratoeva et al. 2017). In my study, the high-fructose high-fat diet increased the kidney mass in male rats but not in female rats (Table 4.6 and Table 4.7). Thus, my findings suggest that male rats were more prone to high-fructose high-fat diet-induced renal harm. Nonetheless, my findings require histological analysis to fully confirm this observation. The hydroethanolic *O. ficus-indica* cladode extract and fenofibrate did not affect kidney mass suggesting that they have decreased potential to cause renal damage. Creatinine, a compound that is produced during muscle damage, is excreted through the kidneys in urine (Motzer et al. 2019). On the other hand, urea is a metabolic by-product of protein metabolism (Salazar 2014). Like creatinine, urea is excreted via the kidneys in urine (Salazar 2014; Motzer et al. 2019). Clinically, an increase in plasma creatinine and urea concentrations signifies impaired renal function (Al Hroob et al. 2018). Thus, creatinine and urea are used as surrogate biomarkers of kidney function (Han et al. 2019; Al Hroob et al. 2018). I report that the high-fructose high-fat diet, hydroethanolic *O. ficus-indica* cladode extract and fenofibrate do not have any effect on the surrogate biomarkers of kidney function in the rats (Figure 4.10 and 4.11). These findings suggest that my treatments did not adversely affect kidney function.

CHAPTER 6: CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

In my study, I assessed the total phenolic content (quantitatively) and phytochemical composition (qualitative) of hydroethanolic *O. ficus-indica* cladode extract as well as its effect in high-fructose high-fat diet-fed growing male and female Sprague-Dawley rats.

Qualitative phytochemical assessments revealed that the hydroethanolic *O. ficus-indica* cladode extract contained bioactive phytochemicals including saponins and tannins. The presence of these bioactive phytochemicals in the hydroethanolic *O. ficus-indica* cladode extract implied that the extract may possess antioxidant, antidiabetic and anti-obesogenic activities. This prompted me to interrogate the potential protective effect of the extract in growing rats fed a high-fructose high-fat diet, mimicking children fed obesogenic diets.

I report that feeding a high-fructose high-fat diet to growing male and female rats for 12weeks resulted in early-stage fatty liver disease that was characterised by increased liver lipid accretion. Due to the fatty-liver disease being in its early developmental stages, the increase in liver lipid accretion did not result in hepatic steatosis, inflammation and damage. The high-fructose high-fat diet increased kidney mass in male rats but did not cause central obesity, dyslipidaemia, insulin resistance or hyperglycaemia in the rats. Moreover, the high-fructose high-fat diet did not adversely impact on the liver and kidney function of the rats. It can be concluded that in growing rats, the high-fructose high-fat diet caused fatty-liver disease and increased kidney mass in a sex-dependent manner, but it did not cause MetS.

My hydroethanolic *O. ficus-indica* cladode extract, at high and low dose, prevented the fatty-liver disease that was induced by the obesogenic diet. Importantly, the hydroethanolic *O. ficus-indica* cladode extract did not jeopardise the kidney and liver function in growing rats fed a high-fructose high-fat diet due to its non-toxicity. Thus, I conclude that my extract exerted anti-lipogenic effects in the rats without compromising their hepatic and renal health. I thus advocate the use of hydroethanolic *O. ficus-indica* cladode extracts as an ethnomedicine against diet-induced fatty-liver disease.

Like the hydroethanolic *O. ficus-indica* cladode extract, fenofibrate prevented the high-fructose high-fat diet-induced fatty liver disease in the rats. However, the fenofibrate resulted in hyperglycaemia; impaired glycaemic control, hepatomegaly and increased plasma ALP activity in growing rats fed a high-fructose high-fat diet. I, therefore, conclude that the use of fenofibrate can result in adverse health outcomes. Thus, fenofibrate should be used with caution.

A limitation of my study is that only qualitative analysis of phytochemical constituents was conducted on the hydroethanolic *O. ficus-indica* cladode extract. Future studies investigating the medicinal properties of plant-derived extracts should conduct quantitative analysis in order to ascertain the main phytochemical constituents. Another limitation of my study is that histological analysis was not conducted the rats' kidneys. Moreover, urea and creatinine are non-specific biomarkers of renal functions. Thus, future studies should conduct histo-morphometric analysis on the kidney and measure specific markers of renal tissue damage, such as kidney injury molecule-1, when evaluating kidney health.

CHAPTER 7: REFERENCES

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APPENDICES

Appendix 1: Animal ethics clearance certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/09/61/B

APPLICANT: Ms AZ Zulu

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Effects og hydroalcoholic Opuntia ficus-indica cladode extract on growing Sprague-Dawley rats fed a high-fructose hight-fat diet

Number and Species

50X 21 day old male Sprague-Dawley rattus norvegicus and 50X 21 day old female Sprague-Dawley rattus norvegicus

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/09/26. This approval remains valid until 2019/10/25.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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:

Signed: _____ (Chairperson, AESC) Date: 2017-10-27

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: 27 October 2017

cc: Supervisor: Dr B Lembede and Professor E Chivandi
Director: CAS

Works 2000/Iain0015/AESCCert.wps

Appendix 2: Rat insulin enzyme-linked immunosorbent assay (ELISA) kit protocol

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

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

Appendix 3: Enzyme-linked calorimetric assay protocol

8th Edition, revised in February, 2018

Application

This kit applies the COD-PAP method and it can be used for *in vitro* determination of total cholesterol (T-CHO) content. It can be operated with all kinds of spectrophotometer.

Detection principle

$$\text{Cholesterol esters} \xrightarrow{CE} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O2 \xrightarrow{CO} \Delta\ 4\text{Cholestenone} + H2O2$$

$$4 - AAP + H202 + 3{,}5 - DHBS \xrightarrow{POD} \text{Red quinone} + H2O$$

The color depth of the generated quinones is directly proportional to the cholesterol content. The absorbance values of the standard tube and the sample tube are measured respectively, and the cholesterol content in the sample can be calculated.

Kit components

Composition	Size	Component	Concentration	Storage
Working Solution (Enzyme)	100 mL × 1 vial	Good's Buffer	50 mmol/L, pH6.7	2-8℃ (shading light)
		Phenol	5 mmol/L	
		4-AAP	0.3 mmol/L	
		Cholesterol esterase	≥ 50 KU/L	
		Cholesterol oxidase	≥ 25 KU/L	
		Peroxidase	≥ 1.3 KU/L	
Standard	0.1 mL × 1 vial	Cholesterol	5.17 mmol/L	

Sample Preparation

1. **Serum (Plasma):** Detect the sample directly. If the concentration is beyond the linear range, then dilute the sample with saline before detection.
2. **Culture fluid sample:** Collect the culture medium, centrifuge at 1000 rpm for 10 min, and take the supernatant for detection.
   [Note]: It is generally recommended that the cell density should be more than 1×10^6 /mL.
3. **Tissue sample:** Accurately weigh the tissue weight, add 9 times the volume of homogenate media according to the ratio of Weight (g): Volume (mL) =1:9. Mechanical homogenate the sample in ice water bath. Centrifuge at 2500 rpm for 10 min, then take the supernatant for detection.
   [Note]: (1) If the tissue sample is not a high-fat sample, the homogenate media should be phosphate buffer (0.1 mol/L, pH 7.4) or normal saline.

(2) If the tissue sample is high-fat sample or partly high lipid sample, the homogenate media should be absolute alcohol.

4. **Cell sample:**

Cell collection: Take the prepared cell suspension and centrifuge at 1000 rpm for 10 min. Discard the supernatant and keep the cell sediment. Wash the sediment with iso-osmia buffer (0.1 mol/L, pH7~7.4 phosphate buffer was recommended) 1~2 times, centrifuge at 1000 rpm for 10 min. Discard the supernatant and keep the cell sediment.

Cell disruption: Add 0.2~0.3 mL of homogenate media (0.1 mol/L, pH7~7.4 phosphate buffer or normal saline was recommended). Sonicate in ice water bath (power: 300 W, 3~5 second/time, interval for 30 sec, repeat for 3~5 times) or grind with hand-operated. The prepared homogenate kept for detection without centrifugation. The cell can also be lysed with the cell lysate buffer (Triton X-100, 1~2%, 30~40 min), then take the prepared lysate for detection directly without centrifugation.

[Note]: It is generally recommended that the cell density should be more than 1×10^6/ml. The disrupted cell can be observed with microscope that whether the cell is broken completely.

Operation table

Operate with test tubes. Colorimetric assay with Spectrophotometer.			
	Blank tube	Standard tube	Sample tube
Distilled water (μL)	10		
Standard (μL)		10	
Sample (μL)			10
Working solution (μL)	1000	1000	1000

Mix thoroughly, incubate at 37℃ for 10 min, set to zero with distilled water and measure the OD value at 510 nm with 0.5 cm diameter cuvette.

Calculation of result

1. **Calculation formula for serum and other liquid sample:**

Total Cholesterol content (*mmol/L*)

$$= \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{Concentration of standard } (mmol/L)$$

2. **Calculation formula for tissue and cells samples:**

Total Cholesterol content (*mmol/gprot*)

$$= \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{Concentration of standard } (mmol/L)$$

÷ Protein concentration of tested sample (*gprot/L*)

Appendix 4: Enzyme-linked fluorescence HDL-cholesterol assay kit protocol

7th Edition, revised in April, 2017

Experimental instrument

Test tube, Micropipettor, Vortex mixer, 37℃ water bath/gas bath, Spectrophotometer (546nm)

Kits components

Reagent composition	Specification	Component	Concentration	Storage
Reagent 1	24 mL×1 vial	Good's Buffer	50 mmol/L	2~8℃ Shading light
		Toos	1 mmol/L	
		$MgCl_2 \cdot 6H_2O$	15 mmol/L	
		Cholesterol oxidase	≥3 KU	
		Peroxidase	≥5 KU	
Reagent 2	8 mL×1 vial	Good's Buffer	50 mmol/L	
		4-amino-antipyrine	0.2 mmol/L	
		$MgCl_2 \cdot 6H_2O$	15 mmol/L	
		Cholesterol esterase	≥3 KU	
		Surfactant	0.1%	
Standard	1 mL×1 vial	Cholesterol	1.05 mmol/L	

Sample treatment

1. **Serum (plasma)**: Detect the sample directly. If the concentration is beyond the linear range, then dilute the sample with saline before detection.
2. **Culture medium sample**: Draw the culture medium, centrifuge at 2500 rpm for 10 min, and take the supernatant for detection.
   [Note]: It is generally recommended that the cell density should be more than 1 million/mL.
3. **Tissue sample**: Accurately weigh the tissue weight, add 9 times the volume of **homogenate media** according to the ratio of Weight (g): Volume (mL) =1:9. Mechanical homogenate the sample in ice water bath. Centrifuge at 2500 rpm for 10 min, then take the supernatant for detection.
   [Note]:
   (1) If there are no high lipid samples exist in the tissue sample, the homogenate media should be extracted with phosphate buffer (0.1 mol/L, pH 7.4) or saline.
   (2) If the tissue sample is high lipid sample or partly high lipid sample, the homogenate media should be extracted with absolute alcohol.
4. **Cell sample**:
   Cell collection: Cell collection: Take the prepared cell suspension and centrifuge at 1000 rpm for 10 min. Remove the supernatant and keep the cell sediment. Wash the sediment with iso-osmia buffer (0.1mol/L, pH7~7.4 phosphate buffer was recommended) 1~2 times, centrifuge at 1000 rpm for 10 min and then remove the supernatant and keep the cell sediment.
   Cell disruption: Add 0.2~0.3 mol/L of homogenate media (0.1 mol/L, pH7~7.4 phosphate buffer or saline was recommended). Sonicate in ice water bath (power: 300W, 3~5 second/time, interval for

30s, repeat for 3~5 times) or grind with hand-operated. The prepared homogenate liquid kept for detection without centrifugation. The cell can also be lysed with the cell lysate buffer (Triton X-100, 1~2%, lysate for 30~40 minutes), then take the prepared homogenate liquid for detection directly without centrifugation.

[Note]:

It is generally recommended that the cell density should be more than 1million/ml. The disrupted cell can be observed with microscope to check that whether the cell is broken completely.

Operation steps

Operate with 96 wells-plate. Colorimetry with Microplate Reader			
	Blank well	Standard well	Sample well
Distilled water (μL)	2.5		
Standard (μL)		2.5	
Sample (μL)			2.5
R1 (μL)	180	180	180
Mix fully and incubate at 37℃ for 5 min. Measure the absorbance value (A1) of each tube at 546 nm with Microplate Reader.			
R2 (μL)	60	60	60
Mix fully and incubate at 37℃ for 5 min. Measure the absorbance value (A2) of each tube at 546 nm wavelength with Microplate Reader.			

Automatic biochemical analyzer operation.	
Sample/Water Volume	2.5 μL
R1	180 μL
Incubate at 37℃ water bath for 5 min. Measure the absorbance of each tube (A_1) at 546 nm with Microplate Reader.	
R2	60 μL
Mix fully and incubate at 37℃ for 5 min. Measure the OD value (A1) of each tube at 546 nm with Microplate Reader.	
Main wavelength	546 nm
Reaction type	Terminal method
Reaction direction	Up reaction (+)