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EFFECT OF DIETARY *TERMINALIA SERICEA* AQUEOUS LEAF EXTRACTS ON HIGH-FRUCTOSE DIET FED GROWING WISTAR RATS

Busisani Wiseman Lembede

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, 2014

DECLARATION

I, **Busisani Wiseman Lembede**, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

……

Busisani Wiseman Lembede

Signed on the……..day of…….2014

DEDICATION

To my late grandmother

Thembeni Alizina Lembede

1928 - 2013

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to the staff of the Central Animal Services, Faculty of Health Sciences, University of the Witwatersrand, for their assistance with the care and welfare of the animals.

Dr. Eliton Chivandi and Associate Professor Kennedy H. Erlwanger: for their supervision and all the valuable knowledge and skills they taught me. They have been good role models and inspired me a lot.

Miss Margaret Badenhorst: for her assistance in preparing the reagents that I used and for the preparing and processing of tissues for histology, and for assistance in determining plasma insulin content using the ELISA technique.

Miss Maphoko Masemola and Mr Mhlengi Magubane: for their assistance with the feeding trial and data collection.

Research, Tuition and Upkeep Funds: I am very grateful to the School of Physiology, Faculty of Health Sciences Research Committee, National Research Foundation and the Oppenheimer Memorial Trust for providing me with financial support to cover my tuition, research and subsistence.

Finally I would like to thank the Creator of all things, God Almighty, for His protection and guidance.

ABSTRACT

Sedentary lifestyles and poor dietary choices are the major cause of the global increase in the prevalence of obesity and metabolic dysfunction in children. The high cost and limited access to conventional drugs by poor communities make them depend on ethnomedicines. *Terminalia sericea (T. sericea)* contains phytochemicals that give its extracts hypolipidaemic and hypoglycaemic properties hence its use in ethnomedicine to treat diabetes mellitus. Using weanling Wistar rat pups fed a high fructose diet to model growing children exposed to high-sugar diets, this study sought to evaluate the effects of aqueous *T. sericea* leaf extracts on their growth performance, glucose homeostasis, visceral morphometry and their general health profile. Forty 21-day old male Wistar pups were randomly allocated to five treatment regimens. Each group had *ad libitum* access to a commercially supplied rat chow. Group 1 pups were given plain drinking water and plain gelatine cubes, group 2: 12% fructose solution and plain gelatine cubes, group 3: 12% fructose solution and gelatine cubes containing fenofibrate at a dosage of 100 $mg.kg^{-1}$ per day, group 4: 12% fructose solution and gelatine cubes with a low dose (100 $mg.kg^{-1}$ per day) of the *T. sericea* extract and group 5: 12% fructose solution and gelatine cubes with a high dose (400 $mg.kg^{-1}$ per day) of the *T. sericea* extract. The pups were maintained on the regimens for 12 weeks after which they under went an oral glucose tolerance test. Fasting blood metabolite content was then determined after which the rats were killed and tissues collected for visceral morphometrical, linear growth and surrogate markers' of health determinations.

T. sericea extracts had no negative effect on growth performance (body mass and indexes of long bone growth) but rats given fenofibrate had lighter empty carcasses and shorter tibiae.

The administration of *T. sericea* extracts neither improved glucose homeostasis nor caused derangement of glucose handling by rats given a high fructose diet following an oral glucose challenge. However, the administration of fenofibrate to rats given a high fructose diet resulted in decreased glucose handling following an oral glucose challenge. With the exception of the administration of fenofibrate which resulted in a significantly high ($P < 0.05$) fasting blood glucose concentration, treatment regimens had no effect on fasting blood glucose, triglyceride and cholesterol concentrations. Rats given fructose with either plain gelatine cubes or low *T. sericea* dose had significantly higher ($P < 0.05$) liver lipid content compared with the control treatment. Administration of *T. sericea* extracts to rats given a high fructose diet had no effect on the GIT, other abdominal viscera and markers of general health. The administration of fenofibrate to rats given a high fructose diet caused increased relative mass of GIT organs (stomach, small intestine and caecum), increased absolute mass of other viscera (liver and kidney); increased serum phosphorus and alkaline phosphatase concentration.

Results from the study revealed that administration of a high dose of aqueous *T. sericea* leaf extracts has potent phytochemicals properties that has helped to prevent high fructose diet-induced deposition of fat in the in the liver (non-alcoholic fatty liver disease), without compromising growth, visceral morphometry and general health of growing Wistar rats.

TABLE OF CONTENTS

EFFECT OF DIETARY *TERMINALIA SERICEA* AQUEOUS LEAF EXTRACTS ON HIGH-FRUCTOSE DIET FED GROWING WISTAR RATS i

DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENTS iv
ABSTRACT v
TABLE OF CONTENTS vii
LIST OF TABLES xi
LIST OF FIGURES xii
LIST OF ABBREVIATIONS xiii
CHAPTER 1: INTRODUCTION 1

1.0 Introduction 2
1.1 Justification of study 3
1.2 Aim of study 6
1.3 Hypothesis 7
CHAPTER 2: LITERATURE REVIEW 8

2.0 Introduction: 9
2.1 Consequences of metabolic syndrome 9
2.1.1 Diabetes mellitus 10
2.1.2 Insulin secretion and complications of type II diabetes mellitus 11
2.2 Models of metabolic syndrome 13
2.2.1 Genetic models 13
2.2.2 Pharmacologically-induced models 14
2.2.3 Diet-induced model 14
2.3 Therapeutic interventions 16
2.4 *Terminalia sericea* 17

2.4.1 Botanical description 17
2.4.2 Taxonomical classification 18
2.4.3 Medicinal uses of *Terminalia sericea* 19
2.4.4 Other uses of *Terminalia sericea* 20
2.5 Chemical composition of *T. sericea* 20
2.5.1 Proximate composition of *T. sericea* seed 20
2.5.2 Lipid profile of *T. sericea* leaves and seeds 21
2.5.3 Phytochemical constituents 21
2.6 Pharmacological actions of *T. sericea* extracts 24
2.6.1 Antimicrobial activity 24
2.6.2 Anti-HIV activity 25
2.6.3 Antioxidant activity 25
2.6.4 Antidiabetic and lipolytic activity 26
2.6.5 Anti-inflammatory 26
2.6.6 Antitumour effect 27
2.6.7 Toxicity 27
CHAPTER 3: MATERIALS AND METHODS 28
3.0 Source of *T. sericea* leaves 29
3.1 Preparation of *T. sericea* leaf extracts 29
3.2 Determination of the phytochemical composition of *T. sericea* leaf extracts 30
3.2.1 Flavonoids 30
3.2.2 Terpenoids 30
3.2.3 Saponins 31
3.2.4 Tannins 31
3.2.5 Anthraquinones 31
3.3 Brine shrimp toxicity assay 32
3.3.1 Hatching of nauplii 32
3.3.2 Brine shrimp assay 32
3.4 Ethical clearance for use of animals and the study site 33
3.5 Animals: feeding and housing 33

3.6 Treatment doses and drug vehicle medium preparation 33
3.7 Treatments and experimental design 34
3.8. Body mass measurement 35
3.9. Experimental intervention and terminal procedures 35
3.9.1 Oral glucose tolerance test 35
3.9.2 Terminal procedures 36
3.10 Statistical analysis 40
CHAPTER 4: RESULTS 42
4.1 Phytochemical composition of aqueous leaf extract 43
4.2 Brine shrimp toxicity assay 45
4.3 Mortality and morbidity of the rats 48
4.4 Effects of aqueous *T. sericea* leaf extract on growth performance 48
4.4.1 Body mass and empty carcass mass 48
4.4.2 Linear growth 53
4.5 Effect of aqueous *T. sericea* leaf extract on glucose tolerance 60
4.6 Effect of aqueous *T. sericea* leaf extracts plasma insulin concentration and HOMA index 65
4.7 Effect of aqueous *T. sericea* leaf extracts on visceral organs 68
4.8 Effect of *T. sericea* aqueous leaf extracts on hepatic metabolic substrates 79
4.9 Effect of aqueous *T. sericea* leaf extract on general health profile 83
CHAPTER 5: DISCUSSION 87
5.1 Phytochemical constituents 88
5.2 *In vitro* toxicity on Brine shrimp nauplii 88
5.3 Growth performance 89
5.3.1 Body mass 89
5.3.2 Linear growth 90
5.4 Glucose tolerance, HOMA-IR and plasma insulin 90
5.5 Visceral organs 93
5.5.1 GIT visceral organs 93
5.5.2 Other viscera 94

5.6 Liver metabolic substrate storage and markers of general health 97
5.6.1 Liver glycogen content 97
5.6.2 Liver lipid content 98
5.6.3 Markers of liver function 99
5.6.4 Markers of kidney function 101
5.6.5 Effect on serum calcium and phosphorus concentrations 102
5.6.6 Effect on serum cholesterol 103
5.6.7 Effect on serum amylase activity 104
CHAPTER 6: CONCLUSION AND RECOMENDATIONS 105
6.0 Conclusion and recommendations 106
6.1 Limitations 107
CHAPTER 7: REFERENCES 109
APPENDICES 135
Appendix 1 136
Appendix 2 137

LIST OF TABLES

Table 4.1 Phytochemical constituents of the aqueous *T. sericea* leaf extract 44

Table 4.2: Effect of aqueous *T. sericea* leaf extract on tibiae and femora lengths, masses and density of the rats 54

Table 4.3 Effect of aqueous *T. sericea* leaf extracts on fasting blood glucose and plasma insulin concentration and HOMA-IR index 66

Table 4.4A: Effect of aqueous *T. sericea* leaf extract on GIT visceral organ masses and lengths in male Wistar rats 69

Table 4.4B: Effect of aqueous *T. sericea* aqueous leaf extract on other visceral organs absolute and relative masses in male Wistar rats 72

Table 4.4C: Effect of aqueous *T. sericea* aqueous leaf extract on villi height, crypt depth and the villi height: crypt depth ratio in male Wistar rats 76

Table 4.4D: Effect of aqueous *T. sericea* leaf extract on hepatocyte size and density of male Wistar rats 77

Table 4.5 The effect of aqueous *T. sericea* leaf extracts on serum markers of general health in male Wistar rats 84

LIST OF FIGURES

Figure1.1: A Schematic representation of the insulin secretion mechanism..... 11

Figure 1.2: The leaves and fruit of *T. sericea*..... 18

Figure 4.1: Toxicity effects of aqueous *T. sericea* extracts on *Artemia salina* (brine shrimp) nauplii. 46

Figure 4.2A: The induction and terminal body masses of the rats given the different treatments 49

Figure 4.2B: Effect of aqueous *T. sericea* leaf extract on the empty carcass mass of the rats..... 51

Figure 4.3: Radiograph images of femora and tibiae of the Wistar rats..... 58

Figure 4.4A: Effect of aqueous *T. sericea* leaf extracts on the glucose tolerance of rats 61

Figure 4.4B: Effect of aqueous *T. sericea* leaf extracts on total area under the curve of oral glucose tolerance of rats 63

Figure 4.5A: Effect of aqueous *T. sericea* leaf extracts on the hepatic glycogen content of the male Wistar rats..... 80

Figure 4.5B: Effect of aqueous *T. sericea* leaf extracts on the hepatic lipid content of male Wistar rats 81

LIST OF ABBREVIATIONS

α:	Alpha
β:	Beta
ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
BM:	Body mass
BUN:	Blood urea nitrogen
COX:	Cyclooxygenase
DM:	Dry matter
FF:	Fenofibrate in gelatine cube
FFAs:	Free fatty acids
FS:	Fructose solution
GIT:	Gastro intestinal tract
HD:	High dose aqueous *Terminalia sericea* leaf extract in gelatine cube
HDL:	High density lipoprotein

HOMA-IR:	Homeostatic model of insulin resistance
IDF:	International Diabetes Federation guidelines
LD:	Low dose aqueous *Terminalia sericea* leaf extract in gelatine cube
LDL:	Low density lipoprotein
LI:	Large intestines
OGTT:	Oral glucose tolerance test
PC:	Plain gelatine cube
PW:	Plain water
PPAR:	Peroxisome proliferator activated receptor
SI:	Small intestine
TLr:	Relative tibia length
TBIL:	Total bilirubin
TNF:	Tumour necrosis factor
VLDL:	Very low density lipoprotein
w/v:	weight/volume
WHO:	World Health Organisation

CHAPTER 1: INTRODUCTION

1.0 Introduction

Urbanisation has led to an increase in the proportion of children and adolescents living sedentary lifestyles and consuming artificially sweetened foods, rich in fructose (Rutledge and Adeli, 2007; Weiss *et al.,* 2004). The consumption of diets high in fructose has been shown to cause obesity and metabolic syndrome in South African adolescents (Steyn and Temple, 2012). Metabolic syndrome is a manifestation of various medical conditions (Weiss *et al.,* 2004) that include abdominal obesity, atherogenic dyslipidaemia, hypoalbuminaemia, raised blood pressure, insulin resistance, glucose intolerance and hypercholesterolaemia (Alberti *et al.*, 2006; Huang *et al.*, 2004). It has been suggested that obesity is the main driver of increased childhood metabolic syndrome, in developing countries (Misra and Khurana, 2008; Kelishadi, 2007). In South Africa the prevalence of childhood obesity is estimated to be 7.8% and it is expected to increase to 22.8% by 2020 (Toriola *et al.,* 2012). The expected increase in childhood obesity will cause an increase in the prevalence of metabolic syndrome as a consequence (Mollentze, 2006).

Pharmaceutical drugs such as metformin and fenofibrates are used to manage metabolic syndrome (Kraja *et al.,* 2010; Kirpichnikov *et al.,* 2002). Despite the existence of pharmaceutical drugs, about 80% of the population in communities in the developing countries rely on medicinal plants to treat ailments such as metabolic dysfunction (Fyhrquist *et al.,* 2002). This is due to the high cost, limited access and availability pharmaceutical drugs to third world country communities. The tree *Terminalia sericea* (*T. sericea*) is widely distributed in subtropical Africa and is used by traditional healers to treat wounds, stomach problems, hypertension and diabetes

mellitus (Mochizuki and Hasegawa 2007; Fyhrquist *et al.,* 2002). Its extracts contain biologically and pharmacologically active phytochemicals including tannins, saponins and flavonoids (Nkobole *et al.,* 2011; Mochizuki and Hasegawa 2006). Tannins, saponins and flavonoids have been demonstrated to have antioxidant, antidiabetic, antimicrobial and cardioprotective properties (Sandhar *et al.,* 2011; Subramaniam *et al.,* 2011; Dwivedi 2007; Eckel *et al.,* 2005). *T. sericea* is used in ethnomedicine to treat diabetes mellitus (Fyhrquist *et al.,* 2002), however only *in-vitro* studies have been done to evaluate its hypoglycaemic, antidiabetic and hypolipidaemic properties (Nkobole *et al.,* 2011; Mochizuki and Hasegawa, 2006).

1.1 Justification of study

Majority of studies evaluating the antidiabetic and antiobesity potential of medicinal plants used streptozotocin-induced diabetic rat models to evaluate the efficacy of the plants (Din *et al.,* 2011; Njomen *et al.,* 2009; Rao and Nammi, 2006). Streptozotocin-induced diabetic rat models do not efficiently mimic the metabolic syndrome in humans caused by the consumption of diets high in refined sugars (Huang *et al.,* 2004) hence it is preferable to use high fructose diet-induced metabolic syndrome rat models (Huang *et al.,* 2004). In addition most studies have used adult rats and have focused on trying to find curative traditional plants (Njomen *et al.,* 2009; Rao and Nammi, 2006). However, the prevalence of metabolic syndrome and obesity has been identified to be increasing in children and adolescents (Kelishadi, 2007) hence the need to use growing animals (i.e growing rats) modelling the development of diet-induced obesity as well as

metabolic dysfunction in children and adolescents (Kohen-Avramoglu *et al.,* 2003) with aim of exploring preventative interventions.

Although there are many conventional pharmaceutical drugs that can be used in treating metabolic syndrome and obesity, their high cost and poor accessibility especially for third world country communities has led to the resurgence of research focusing on indigenous trees and herbs as potential treatments for metabolic syndrome (Ayyanar *et al.,* 2008). *T. sericea* is an indigenous tree with multiple ethnomedicinal uses (Deutschlander *et al.,* 2009; Mochizuki and Hasegawa 2007) including the treatment of diabetes mellitus (Deutschlander *et al.,* 2009; Fyhrquist *et al.,* 2002). *In vitro* studies have reported that *T. sericea* stem bark and root to have anti-diabetic and anti-obesity properties (Nkobole *et al.,* 2011; Mochizuki and Hasegawa 2006). *T. sericea* aqueous leaf extracts contain saponins, tannins and flavonoids (Masoko *et al.,* 2005; Bessong *et al.,* 2004; Fyhrquist *et al.,* 2002). Saponins, tannins and flavonoids are known to have hypoglycaemic, anti-diabetic and lipolytic properties thus *T. sericea* could have the potential to prevent the onset of fructose-induced metabolic dysfunction and obesity. There is little or no research that has been done regarding *T. sericea* leaves as a potential preventative medication for high fructose diet-induced metabolic dysfunction and obesity in growing rats modelling human children and adolescents.

The first point of contact for orally administered substances is the gastrointestinal tract (GIT) (Rubino *et al.,* 2010). Therefore, the first place where the plant extracts exert their effects would be in the GIT. The ontogenic development of the GIT has three-stages: the prenatal, neonatal and

post-weaning stage (Zabielski *et al.,* 2008). Prenatally there is little stimulation of the GIT lumen (Zabielski *et al.,* 2008). Neonatal stimulation is by milk and the post-weaning stage is when the switch from milk to solid feed occurs (Zabielski *et al.,* 2008). The post-weaning stage is a critical stage in the development of the GIT. The introduction of solid feeds too early (to the not yet adapted GIT of infants) increases the risk of developing GIT post-weaning disorders (Zabielski *et al.,* 2008). The GIT is vital in the digestion and absorption of nutrients; altering nutrient absorption can affect overall growth and development of the growing animal (Pérez *et al.,* 2007).

Compounds found in plant extracts used in ethnomedicines can exert negative or positive effects on the nutrient absorptive capacity of the GIT (González-Alvarado *et al.,* 2007; Kochhar *et al.,* 2007). *T. sericea* leaves are rich in tannins (Nkobole *et al.,* 2011). Studies have suggested that tannins may have detrimental effects on the mucosal layer of the GIT, resulting in reduced nutrient absorption and reduced growth in animals (Chung *et al.,* 1998). However, Mochizuki and Hasegawa (2007) showed that *T. sericea* sericosides have protective properties within the GIT lumen. Thus, administration of *T. sericea* orally can potentially have positive or negative effects on the development of the GIT, as well as nutrient absorption. Furthermore, medicinal plants used in communities are usually folk referrals and are believed to be harmless to the body (Erasto *et al.,* 2005). However, medicinal plants have the potential to cause adverse side effects such as cytotoxicity (Bussmann *et al.,* 2011; Kane *et al.,* 1995). Hence, when researching medicinal properties of plants there is a need to also screen them for toxicity.

1.2 Aim of study

The broad objective of the current study was to determine the effects of orally administered aqueous *T. sericea* leaf extracts in high-fructose diet-fed growing Wistar rats. Specifically the study sought to determine the effects of orally administered aqueous *T. sericea* leaf extracts in growing Wistar rats on:

a. growth performance (body mass and indexes of long bone growth).
b. glucose tolerance.
c. serum concentrations of metabolic substrates (glucose, triglycerides, free fatty acids and cholesterol).
d. serum insulin concentration and HOMA IR index.
e. liver glycogen and lipid storage.
f. the general health profile by determining serum activities of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) and other markers (serum creatinine, urea, total bilirubin, globulin, calcium, phosphorus, total protein, albumin, cholesterol, and amylase) of general health.
g. GIT and visceral organ macro- and micro-morphometry.
h. To determine the phytochemical composition and toxicity of aqueous *T. sericea* leaf extracts, using qualitative phytochemical screening and the brine shrimp toxicity assay.

1.3 Hypothesis

The null and alternate hypotheses of the study were:

i. H_0: Orally administered aqueous *T. sericea* leaf extracts do not improve the growth performance, post-weaning GIT development and have no effect on markers of metabolic dysfunction and the general health profile of growing male Wistar rats given a high fructose diet.

ii. H_1: Orally administered aqueous *T. sericea* leaf extracts improve growth performance, post-weaning GIT development and affect markers of metabolic dysfunction and the general health profile of growing male Wistar rats given a high fructose diet.

CHAPTER 2: LITERATURE REVIEW

2.0 Introduction:

Metabolic syndrome refers to the manifestation of a combination of risk factors (visceral obesity, insulin resistance, hyperglycaemia, hypertriglyceridaemia, increased low density lipoprotein, decreased high density lipoprotein and hypertension) that increase the chance of developing type II diabetes mellitus and cardiovascular diseases (Beilby, 2004). According to the International Diabetes Federation guidelines (IDF) and World Health Organisation (WHO) guidelines, metabolic syndrome in children and adolescents is diagnosed when patients present with central obesity and any other 2 of hyperglycemia, hypertriglyceridaemia, hypertension and decreased high density lipoprotein (Friend *et al.,* 2013). In developing countries the number of children and adolescents that are obese has been increasing rapidly thus more children and adolescents are at risk of developing metabolic dysfunction (Al-Isa, 2013). Metabolic dysfunction is associated with altered insulin secretion, insulin resistance and type II diabetes mellitus (Al-Isa, 2013).

2.1 Consequences of metabolic syndrome

Insulin resistance, an inability of target cells to respond appropriately to insulin (Mather *et al*., 2007), and hyperglycaemia are the main mediators of the consequences of metabolic syndrome. It (insulin resistance) results in hyperglycaemia which leads to dyslipidaemia (Gall *et al.,* 2010). The latter is characterised by higher than normal plasma concentrations of triglycerides, cholesterol and low density lipoproteins (Li *et al.,* 2011) which is driven by the greater esterification of free fatty acids (FFAs) compared to their oxidation (Thirunavukkarasu *et al.*,

2004). Increased esterification of FFAs promotes the formation and secretion of triglycerides, cholesterol and very low density lipoprotein (VLDL) by the liver (Rutledge and Adeli, 2007; Thirunavukkarasu *et al.,* 2004). Very low density lipoprotein (VLDL) is known to be inefficient at clearing plasma cholesterol (Thirunavukkarasu *et al.,* 2004), it transports the cholesterol from the liver to skeletal and cardiac muscle as well as adipose tissue (Choi and Ginsberg, 2011). The increase in triglyceride, cholesterol and non-high density lipoproteins increases inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Wajchenberg *et al.,* 2009). An increase in inflammatory cytokines combined with dyslipidaemia, hyperglycaemia and insulin resistance can lead to the onset type II diabetes mellitus and its complications (LaRosa *et al.,* 2013; Matsudaa and Shimomurab, 2013).

2.1.1 Diabetes mellitus

Worldwide, diabetes mellitus is ranked as the third most common chronic disease in children (Pettitt *et al.,* 2013). Diabetes mellitus is a metabolic disorder charecterised by increased blood glucose concentration (Pettitt *et al.,* 2013). The two most common subtypes of diabetes mellitus are Type I and II diabetes mellitus. Type I diabetes occurs due to autoimmune destruction of β-cells of the pancreas (Herold *et al.,* 2002) while type 2 occurs due to altered insulin secretion and resistance by body tissue (Neergheen-Bhujun *et al.,* 2013). In 2011, an estimated 366 million people had diabetes mellitus worldwide. This figure is expected to increase to 552 million by 2030 (Al-Sarihin *et al.,* 2013). In sub-Suharan Africa, about 12.1 million people had diabetes mellitus

(Chen *et al.*, 2011a), with an estimated increase to 23.9 million by 2030 (Chen *et al.*, 2011a; Mbanya *et al.*, 2010).

2.1.2 Insulin secretion and complications of type II diabetes mellitus

Insulin is a peptide hormone produced by the β-cells of the endocrine pancreas (Inzucchi and Sherwin, 2011). It is a major anabolic hormone that regulates metabolism (Dentin *et al.*, 2007). It promotes the uptake and storage of glucose and lipids in the liver, muscle and adipose tissue (Qu *et al.*, 2011). The secretion of insulin is stimulated by increase blood glucose concentration (Qu *et al.*, 2011). The mechanism of insulin secretion is as summarised in Figure 1.1 below.

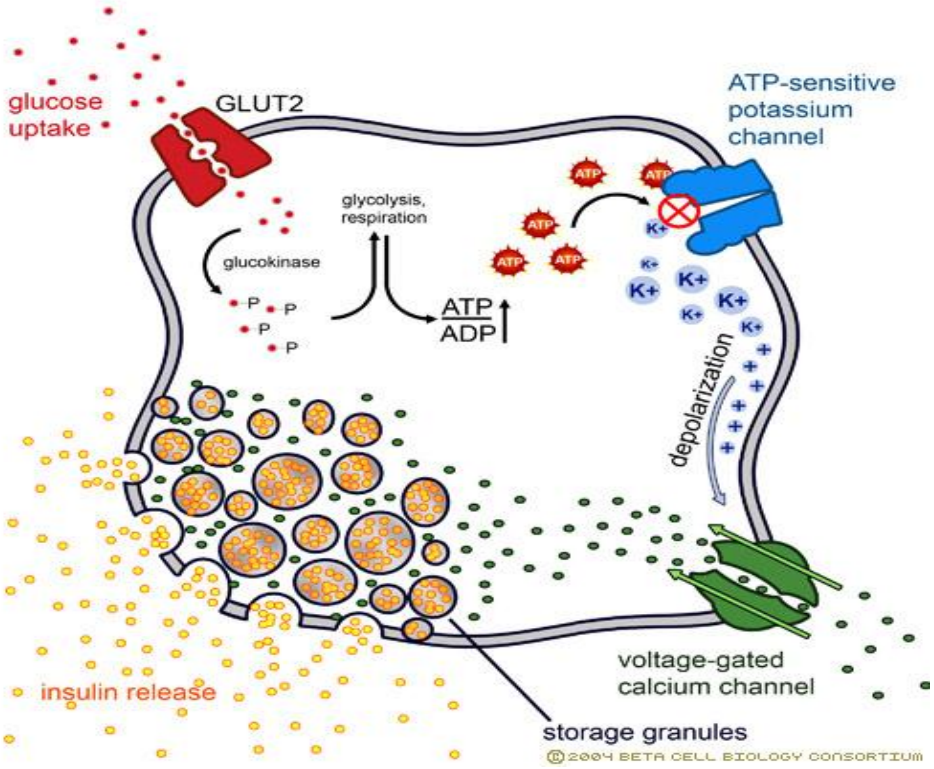

Figure1.1: A Schematic representation of the insulin secretion mechanism

Glucose enters the β-cell from the blood by facilitation of GLUT 2 transporters (Scheepers *et al.,* 2004). The increased generation of ATP via glycolysis inside β-cells of endocrine pancreas causes the potassium channels to close resulting in an influx of calcium which depolarises the cells and subsequently secretes insulin by the β-cells (Scheepers *et al.,* 2004). Source: Beta Cell Biology Consortium, 2004; (www.betacell.org/content/articleview/article_id/1/).

In patients with type II diabetes the secretion and action of insulin as well as glycaemic control are altered leading to microvascular and macrovascular complications (Donaghue *et al.,* 2009). Diabetic retinopathy, a microvascular complication of type II diabetes mellitus, affects 90% of diabetic patients (Rotimi *et al.,* 2003). Altered glycaemic control causes progressive retinal vessel damage and can ultimately lead to blindness (Cheung *et al.,* 2007; Rotimi *et al.,* 2003). Diabetic nephropathy, another microvascular complication in diabetic patients, causes kidney damage that can result in renal failure (Donaghue *et al.,* 2009). Macrovascular complications of type II diabetes mellitus include coronary heart disease, peripheral vascular disease and stroke (Donaghue *et al.,* 2009). Coronary heart disease is the major cause of death in type II diabetic patients (Hurst and Lee, 2003). Atherosclerosis which causes the stiffening and narrowing of arteries as a result of the formation of lipid plaques in the arterial intima is associated with the development of coronary heart disease, stroke and hypertension in diabetic patients (Southerland *et al.,* 2012).

Although diabetes mellitus and metabolic syndrome have been well investigated and documented using humans and animal models, there still is a need to further investigate ways of preventing

and managing diabetes mellitus, as well as developing experimental models of diabetes mellitus and metabolic syndrome.

2.2 Models of metabolic syndrome

The rise in incidence of the metabolic syndrome in children, adolescents and adults has led to the development of various research models with the aim of investigating the mechanisms involved in the development of metabolic syndrome. The models in use include genetically, chemically and diet-induced rat models (Wu *et al.,* 2007; Russell and Proctor, 2006; Bertram and Hanson, 2001).

2.2.1 Genetic models

Genetic models of components of metabolic syndrome are based on monogenic mutation (Bertram and Hanson, 2001) and include the Zucker diabetic fatty rats (fa/fa), db/db (C57BL/KsJ-db/db) mice, ob/ob (C57BL/6J-ob/ob) mice, Goto-Kakizaki rats and the Otsuka Long-Evans Tokushima fatty rats (Panchal and Brown, 2011; Bertram and Hanson, 2001). The rat models are suitable for evaluating the single gene mutation molecular mechanisms involved in the metabolic syndrome (Panchal and Brown, 2011). In humans metabolic syndrome is a multi-factorial disease therefore monogenic rat models are not suitable in mimicking metabolic syndrome seen in humans (Lehnen *et al.,* 2013; Buettner *et al.,* 2006). There are newer models of metabolic syndrome that are polygenic (Morton *et al.,* 2005). However these polygenic models

are also only suitable for studying the specific gene mutation induced components of metabolic syndrome.

2.2.2 Pharmacologically-induced models

Chemical models of metabolic syndrome are developed using alloxan or streptozotozin (Szkudelski, 2001). Alloxan and streptozotocin are pyramidine glucose analogues which selectively destroy the endocrine pancreatic β-cells (Panchal and Brown, 2011; Lenzen, 2008). The models in which high doses are used are well suited for investigating type I diabetes mellitus, whilst the use of low doses is suited for type 2 diabetes mellitus (Zhang *et al.,* 2008b). In an effort to include the dietary complications, researchers have developed models using a combination of streptozotocin at low doses and high fat or high fructose diets (Wilson and Islam, 2012; Bell *et al.,* 2000).

2.2.3 Diet-induced model

Fat, sucrose and fructose are used as dietary ingredients to induce metabolic syndrome in rat models (Lehnen *et al.,* 2013; Busserolles *et al.,* 2003). High-fructose diet rat models of metabolic syndrome are touted as the best models to use when studying diet-induced metabolic syndrome in humans (Oron-Herman *et al.,* 2008), as they cause the development of diverse complications seen in humans with diet induced metabolic syndrome (Lehnen *et al.,* 2013).

2.2.3.1 Fructose-induced metabolic syndrome: mechanism(s) involved

Fructose is a monosaccharide that requires glucose transporters (Glut 5) to aid its absorption from the jejunum (Miller and Adeli, 2008). Once absorbed from the jejunum, fructose is transported to the liver where fructose-1-phosphatase mediates its metabolism to 3-carbon molecules: glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Rutledge and Adeli, 2007). These 3-carbon molecules are used to form triglycerides and free fatty acids (Miller and Adeli, 2008). Unlike glucose that activates phosphofructokinase, fructose activates fructose-1-phosphatase (Rutledge and Adeli, 2007). Phosphofructokinase is a rate limiting enzyme that will inhibit formation of triglycerides from glucose, promote glycogen formation from glucose and stimulate insulin secretion (Woo *et al.,* 2010). This means increased absorption of fructose translates into increased lipogenesis (Basciano *et al.,* 2005).

Lipogenesis due to high fructose diet consumption results in increased visceral obesity which promotes the synthesis and release of tumour necrosis factor alpha (TNF-α) and C-reactive protein peptides (Matsudaa and Shimomurab, 2013; Wajchenberg *et al.,* 2009). These inflammatory mediators inhibit Glut-4 transporter protein mRNA formation hence decreasing the overall amount of Glut-4 receptors which are important for insulin-dependent glucose uptake by tissues (Hotamisligil *et al.,* 1994). Decreased Glut-4 receptors lead to inhibition of insulin-activated glucose uptake (Jellinger, 2007), depriving them (cells) of the primary source of energy leading to their dependence on lipids (secondary) and protein (tertiary) energy stores (Hsu *et al.,* 2012). The utilisation of these stores results in elevated plasma triglycerides, free fatty acids and a negative nitrogen balance (Hsu *et al.,* 2012). Increased triglycerides and free fatty acids can over-stimulate

the pancreas causing increased insulin secretion and later on resulting in lipotoxicity of the pancreatic β-cells (Huang *et al.,* 2004). More importantly elevated triglycerides and free fatty acids will lead to increased plasma low density lipoproteins, cholesterol and oxidative stress (Wajchenberg *et al.,* 2009).

Pharmaceutical drugs such as fenofibrate, metformin, statins, thiazolidinediones and sulfonylurea are used to manage the consequences of metabolic syndrome (Florez *et al.,* 2013; Jialal and Smith, 2012).

2.3 Therapeutic interventions

Fenofibrate, one of the many therapeutic drugs used to manage metabolic syndrome, is a hypolipidaemic pharmaceutical drug that lowers cholesterol, triglycerides and low density lipoprotein in patients at risk of developing cardiovascular diseases (Ling *et al.,* 2013). A derivative of fibric acid, it has a half-life of 20 hours (Tsimihodimos *et al.,* 2005) and acts by activating the peroxisome proliferator-activated receptors alpha (PPARα) ligand, thus activating genes that regulate lipoprotein lipase, acyl-Co-A oxidase and carnitine palmitoyltranferase (Jeong *et al.,* 2004b). Lipoprotein lipase promotes the hydrolysis of plasma triglycerides, whilst acyl-Co-A oxidase and carnitine palmitoyltranferase promote β-oxidation of free fatty acids, and results in inhibition of low density lipoprotein formation (Kraja *et al*., 2010; Tsimihodimos *et al.,* 2005). Fenofibrate also lowers cholesterol by preventing its absorption from the small intestinal lumen into the lymph and by promoting HDL formation (Valasek *et al.,* 2007), which (HDL) clears

circulating cholesterol. The use of fenofibrate is approved for children and adolescents and is known to cause similar beneficial and detrimental effects as seen in adults (Singh, 2006; Steinmetz *et al.,* 1981).

While pharmaceutical drugs such as fenofibrate can be used to manage metabolic dysfunction, they are costly and inaccessible to most third world country communities. Traditional medicinal plants including *Terminalia sericea* (*T. sericea*) thus provide valuable, accessible and affordable remedies to 80% of the third world country communities (Fyhrquist *et al.,* 2002).

2.4 *Terminalia sericea*

T. sericea also known as silver cluster-leaf, and locally as vaalboom, mogonono, moxonono, mususu, amangwe, mangwe, mpululu and namatipo (Deutschlander *et al.,* 2009), is widely distributed in sub-tropical Africa (Joseph *et al.,* 2007; Fyhrquist *et al.,* 2002). It (*T. sericea*) contains bioactive compounds and is used in ethnomedicine to treat various systemic ailments in African communities (Eldeen *et al.,* 2006).

2.4.1 Botanical description

T. sericea is a medium sized deciduous tree that grows up to 9m (Chivandi *et al.,* 2013). Its bark is dark grey, thick, fibrous and is longitudinally fissured (Deutschlander *et al.,* 2009). The leaves of *T. sericea* are concentrated on the tips of the shoot and are covered in cottony silver hairs

(Deutschlander *et al.*, 2009). The flowers are a pale-yellow or creamy white colour (Chivandi *et al.*, 2013). The oval shaped *T. sericea* fruits are a purplish-red colour when immature and turn brown when they mature (Chivandi *et al.*, 2013).



Figure 1.2: The leaves and fruit of *T. sericea*

2.4.2 Taxonomical classification

T. sericea belongs to the Kingdom Plantae, Division Magnoliophyta, Class Magnoliopsida, Order Myrtales, Family Combretaceae, Genus *Terminalia* and Species s*ericea* (Anderson and Bell, (1974).

2.4.3 Medicinal uses of *Terminalia sericea*

2.4.3.1 Leaves

Leaves of *T. sericea* are used to treat bacterial infections (Mochizuki and Hasegawa, 2007). Dried leaves are used to treat intestinal infections that cause diarrhoea (Mochizuki and Hasegawa, 2007). In Venda, South Africa, the dried leaf extract of *T. sericea* is used to manage menorrhagia while the dried leaf powder is used to treat infected wounds (Moshi and Mbwambo, 2005; Fyhrquist *et al.,* 2002). A decoction of *T. sericea* plant is used to treat sexually transmitted diseases and virility/infertility in males (Moshi and Mbwambo, 2005; Arnold and Gulumian, 1984).

2.4.3.2 Roots

Hot root infusions are used to treat pneumonia (Deutschlander *et al.,* 2009) and bacterial infections (Mochizuki and Hasegawa 2007). In Tanzania the roots are used to manage headaches, backaches, diabetes mellitus, diarrhoea and gonorrhoea (Moshi and Mbwambo, 2005). In East Africa root decoctions of *T. sericea* have diuretic properties and are used to treat hypertension, bilharziasis and stomach ailments (Fyhrquist *et al.,* 2002).

2.4.3.3 Fruit and bark

The *T. sericea* fruits are used to treat tuberculosis in Malawi (Moshi and Mbwambo 2005; Fyhrquist *et al.,* 2002). The stem bark is used to treat diabetes mellitus (Deutschlander *et al.,* 2009).

2.4.4 Other uses of *Terminalia sericea*

Terminalia sericea extracts rich in sericoside are processed and used to produce skin penetrating topical cream (Moshi and Mbwambo 2005; Rode *et al.,* 2003). Its wood is used as a fuel energy source and building material (Shackleton, 2001). *T. sericea* plants produce edible gum for humans and serve as a source of foraging to browsing herbivores (Aganga *et al.,* 2000; Davidson 1998).

2.5 Chemical composition of *T. sericea*

Different parts of *T. sericea* are composed of various chemical compounds including; phytochemicals, fibre, minerals, vitamins, amino acids and free fatty acids (Chivandi *et al.,* 2013; Nkobole *et al.,* 2011; Davidson, 1998).

2.5.1 Proximate composition of *T. sericea* seed

The *T. sericea* seeds contain 46.2% crude protein and 32.6% lipid (Chivandi *et al.,* 2013). They also contain minerals including; phosphorus (1121.75±10.39 mg.100g^{-1} DM), calcium

(795.20±17.82 mg.100g^{-1} DM) and magnesium (560.70±6.68 mg.100g^{-1} DM) (Chivandi *et al.*, 2013). Chivandi *et al.* (2013) reported that *T. sericea* seeds contain amino acids with glutamic acid (8.07±0.13 g.100g^{-1} DM) being the most concentrated amino acid. The seeds contain 223.30±2.16 g.kg^{-1} DM and 90.27±4.68 g.100g^{-1} DM neutral and acid detergent fibre, respectively (Chivandi *et al.*, 2013).

2.5.2 Lipid profile of *T. sericea* leaves and seeds

T. sericea leaves and seeds contain saturated, monosaturated and polysaturated fatty acids (Chivandi *et al.*, 2013; Davidson, 1998). The most concentrated monosaturated and polysaturated fatty acids in *T. sericea* seed are oleic and α-linolenic, respectively (Chivandi *et al.*, 2013). Whilst total lipid content of *T. sericea* leaves is higher during the dry season than the wet season (Davidson, 1998), the saturated and monoenoic fatty acids are highest during the dry season and oleic and alpha-linolenic fatty acids are highest during the wet and transition season (Davidson, 1998). The presence of linoleic acid in the leaves and seeds of *T. sericea* is suggestive of its potential herbal use as an antioxidant and for the management of metabolic dysfunction (Racine *et al.*, 2010; Zhang *et al.*, 2008a).

2.5.3 Phytochemical constituents

Tannins, triterpernoid saponins and flavonoids are found in the leaves, bark and roots of *T. sericea* (Nkobole *et al.*, 2011; Mochizuku & Hasegawa, 2006; Bessong *et al.*, 2004). The

triterpernoid saponin is made up of sericoside, arjunglucoside and sericic acid (Eldeen *et al.*, 2006; Rode *et al.,* 2003). Anolignan B, termilignan B and arjunic acid are some of the phytochemicals that have been recently isolated in *T. sericea* ethyl acetate extracts (Eldeen *et al.*, 2008). Some of these phytochemicals have been identified as the compounds that give *T. sericea* its antimicrobial activity (Eldeen *et al.,* 2008; Eldeen *et al.,* 2006).

2.5.3.1 Tannins

Tannins are naturally occurring polyphenols found in various fruits, vegetables and plants (Chung *et al.,* 1998), that have antioxidant properties and lower blood glucose and lipid content (Hagerman *et al.,* 1998). They reduce carbohydrate and lipid digestion as well as absorption by inhibiting amylases and lipases, respectively (Chung *et al.,* 1998). Tannins derived from *Terminalia* have been demonstrated to promote wound healing (Chaudhari and Mengi, 2006), decrease visceral fat, lower blood pressure and prevent free radical induced cardiotoxicity (Singh *et al.,* 2008; Dwivedi, 2007); these properties justify the use of *T. sericea* in ethnomedicine to treat diabetes, hypertension and wounds. *In vitro* studies indicated that tannins can cause deterioration of the mucosal lining of the GIT, increase excretion of essential proteins and decrease growth performance in mammals, impair glucose transport and decrease vitamin A absorption in the intestines (Chung *et al.,* 1998). However *in vivo* studies have indicated contradictory results, showing that tannins have no effect on GIT morphology and excretion of essential amino acids (Chung *et al.,* 1998).

2.5.3.2 Saponins

Saponins are glycosides that occur naturally in plants and form soap-like foam in water (Wina *et al.,* 2005). Triterpenoid saponins, namely lupeol, resveratrol-3-o-B-rutinoside, resveratrol, stigmasterol, B-sitoster have been extracted from *T. sericea* root and bark extracts (Nkobole *et al.*, 2011; Joseph *et al.,* 2007; Moshi & Mbwambo, 2005). Saponins decrease blood glucose, cholesterol and low density lipoprotein concentration in humans (Mahato, 1988). It is hypothesised that *T. sericea* derived saponins lower blood glucose and cholesterol content by stimulating the endocrine pancreatic β-cells to secrete insulin and by inhibiting glucose and cholesterol absorption from the GIT (Subramaniam *et al.,* 2011**;** Francis *et al.,* 2002). Saponins have been demonstrated to inhibit glucose uptake in Ehrlich ascites tumour cells (Hasegawa 1994) and thus inhibit growth of tumour cells, probably by depriving them of nutrients (Francis *et al.,* 2002). Furthermore, saponins have scavenging properties that allow them to protect cells and cell membranes from damage by free radicals (Eckel *et al.,* 2005). The presence of saponins in *T. sericea* leaves is a probable indicator of its' extracts to be used in the management of metabolic dysfunction. Saponins of *T. sericea* have been demonstrated to have anti-inflammatory activity (Eldeen *et al.,* 2005). Treatment of hypophysial cells with saponins led to increased production of luteinising hormone by the cells (Francis *et al.,* 2002). The latter increases production of testosterone by testes which is responsible for the development secondary male sex characteristics hence this may justify the use of *T. sericea* in ethnomedicine to treat virility/infertility men.

2.5.3.3 Flavonoids

Flavonoids are polyphenolic compounds found in plants (Tapas *et al.,* 2008) and are well known for their antioxidant activity. Flavonoids, namely rutinoside, catechin, epicatechin, gallocatechin and epigallocatechin have been extracted from *T. sericea* stem bark (Nkobole *et al.,* 2011). Free radicals that are bound to flavonoids cannot cause cellular damage (Sandhar *et al.,* 2011). The antioxidant and anticoagulant properties of flavonoids prevent vessel endothelial dysfunction, thrombosis and cardiovascular diseases (Sandhar *et al.,* 2011). Flavonoids stimulate insulin release and pancreas β-cells regeneration (Tapas *et al.,* 2008). They also promote hepatocyte regeneration by increasing activity of RNA polymerase I (Tapas *et al.,* 2008), thus suggesting that they have potential to treat liver disease. Additionally, flavonoids are cyclooxygenase (COX) inhibitors and they can potentially be used as anti-allergy and anti-inflammatory compounds (Tapas *et al.,* 2008).

2.6 Pharmacological actions of *T. sericea* extracts

2.6.1 Antimicrobial activity

Triterpene saponins, tannins and flavonoids are toxic to microorganisms; they bind to microbial cell membranes and impair their functioning (Bowyer *et al.,* 1995; Scalbert, 1991). Both organic and aqueous decoctions of *T. sericea* exhibit antimicrobial activity (Eldeen *et al.,* 2008; Eldeen *et al.,* 2006; Moshi and Mbwambo, 2005; Fyhrquist *et al.,* 2002). *T. sericea* organic and aqueous root, stem bark and leaf extracts, *in vitro* exhibit anti-bacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Enterobacter aerogenes, Bacillus*

subtilis, Micrococcus luteus, Escherichia coli, Klebsiella pneumonia and *Sarcina specie* (Eldeen *et al.,* 2008; Eldeen *et al.,* 2006; Moshi and Mbwambo, 2005; Fyhrquist *et al.,* 2002). They also exhibit antifungal activity against *Candida albicans, Aspergillus niger, Aspergillus fumigates, Cryptococcus neoformans, Microsporum canis* and *Sporothrix schenckii* (Eldeen *et al.,* 2006; Moshi and Mbwambo, 2005; Masoko *et al.,* 2005; Steenkamp *et al.,* 2004; Fyhrquist *et al.,* 2002). The antimicrobial activity of *T. sericea* extracts is said to be due to the presence of triterpene saponins, tannins, flavonoids and anolignan B (Eldeen *et al.,* 2006; Fyhrquist *et al.,* 2002).

2.6.2 Anti-HIV activity

Tannins, flavonoids, triterpene saponins, anolignan A and anolignan B are thought to be the compounds that give *T. sericea* its anti-HIV properties (Tshikalange *et al.,* 2008; Wall *et al.,* 1996). *In vitro* studies have reported that organic and aqueous *T. sericea* leaf and root extracts have inhibitory properties against human immunodeficiency virus (HIV-1) replication enzymes (Tshikalange *et al.,* 2008; Bessong *et al.,* 2004).

2.6.3 Antioxidant activity

Studies have reported that *T. sericea* aqueous and methanolic stem bark extracts have antioxidant activity (Nkobole *et al.,* 2011; Steenkamp *et al.,* 2004). *T. sericea* aqueous and methanolic bark extracts significantly lowered oxidant production in formyl-methionyl-leucyl-phenylalanine

stimulated neutrophils (Steenkamp *et al.,* 2004). The antioxidant properties of *T. sericea* stem bark extracts are said to be due to triterpene saponins tannins and flavonoids, which scavenge harmful oxidants (Nkobole *et al.,* 2011; Eckel *et al.,* 2005).

2.6.4 Antidiabetic and lipolytic activity

Nkobole *et al.* (2011) tested the anti-diabetic activity of *T. sericea* stem bark by measuring inhibitory activity of alpha-glucosidase and alpha amylase in an *in vitro* bioassay. *T. sericea* stem bark extracts showed strong inhibition of alpha-glucosidase and alpha-amylase, thus suggesting that it may have anti-diabetic properties. Similarly, Tshikalange *et al*. (2008) reported inhibitory activity *T. sericea* chloroform, ethyl-acetate and 70% acetone bark extracts on alpha-glucuronidase and beta-glucuronase. However, an *in vivo* study by Moshi and Mbwambo (2005) reported that administering a single dose (200 $mg.kg^{-1}$ body mass) of 20% aqueous-ethanol *T. sericea* root extracts had no effect on glucose tolerance of Wistar mice.

2.6.5 Anti-inflammatory

Eldeen *et al.* (2005) reported that ethyl acetate, ethanol and aqueous root and stem bark extracts of *T. sericea* inhibited cyclooxygenase-1, an enzyme key to inflammatory responses. Central obesity can induce an inflammatory response that results insulin resistance (Hotamisligil *et al.,* 1994). Mochizuki and Hasegawa (2007) tested *T. sericea* roots for their protective effect against 2,4,6-trinitrobenzesulfonic acid (TNBS) induced inflammatory bowel disease (IBD). The *T.*

sericea sericoside in the roots of *T. sericea* was reported to have protective effects against TNBS induced IBD (Mochizuki and Hasegawa, 2007). Additionally, flavonoids and saponins, present in *T. sericea* extracts have been reported to mitigate inflammation by suppressing mediators of inflammation (Eldeen *et al.,* 2008; Eldeen *et al.,* 2006; Eldeen *et al.,* 2005).

2.6.6 Antitumour effect

T. sericea leaf and root extracts have been shown to be cytotoxic to T 24 bladder cancer cell, Hela cervical cancer cells and MCF breast cancer cells and thus inhibiting their proliferation (Fyhrquist *et al.,* 2006; Bessong *et al.,* 2004). Tannins, saponins and flavonoids, which are present in *T. sericea* extracts, have been implicated as being anti-cancerous agents (Fyhrquist *et al.,* 2006; Bessong *et al.,* 2004).

2.6.7 Toxicity

Plants contain phytochemicals that have the potential to be toxic. It is important to evaluate the toxic potential carried by traditional plants. Cytotoxicity of *T. sericea* dichloromethane, ethyl acetate, dichloro:methanol, methanol and ethanol root extracts, have been investigated using standard brine shrimp toxicity tests. It was reported that all extracts showed toxicity but dichloromethane and petroleum extracts being the exceptions (Moshi and Mbwambo, 2005). Bessong *et al.* (2004) reported that *T. sericea* aqueous and methanolic extracts showed no toxicity in Hela P4 cell lines that were treated with 600 $\mu g.ml^{-1}$ of the extracts.

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CHAPTER 3: MATERIALS AND METHODS

3.0 Source of *T. sericea* leaves

T. sericea leaves were collected in the summer of 2012 at Maubane N1 toll road, Republic of South Africa. The area on latitude 25° 16' 34.13", longitude 28° 17' 55.92" has an average annual maximum temperature of 27ºC, mean annual rainfall of 530 mm-650 mm (M'marete, 2003). The area (Maubane) is characterised by black clay soils (Coetzee *et al.*, 2003).

The identification of the collected leaves was authenticated by a botanist (Mr Donald McCallum) at the University of the Witwatersrand's Herbarium. A sample of the *T. sericea* plant was stored at the University of the Witwatersrand Herbarium under the reference number J96241.

3.1 Preparation of *T. sericea* leaf extracts

The leaves were dried overnight in an oven (Salvis ®, Salvis Lab, Switzerland) at 40°C (Ahmed *et al.*, 2005), after which they were ground to a course powder in a blender (Waring®, Lasec SA, Johannesburg, South Africa). The extraction was done using distilled water. Briefly, every 25 g of the leaf powder was mixed with 250 ml of boiling distilled water and placed on a shaker for 24 hours (Bessong *et al.*, 2004). The mixture was filtered twice: firstly through a cheese cloth and thereafter through filter paper (Whatmann®, No 1 size 50 mm, England) (Bessong *et al.*, 2004). The resultant filtrate was concentrated by evaporating at 50°C in a rotor vacuum evaporator [Labocon (Pty) Ltd, Krugersdorp, South Africa] and then placed in an oven (Salvis ®, Salvis Lab,

Switzerland) at 40°C till they reached dryness after four days. The dry extracts were stored in air tight dark glass bottles and kept in a dark cupboard until use.

3.2 Determination of the phytochemical composition of *T. sericea* leaf extracts

Qualitative tests were used to determine the presence and or absence of flavonoids, terpenoids, saponins, tannins and anthraquinones in *T. sericea* aqueous leaf extracts.

3.2.1 Flavonoids

The presence of flavonoids was determined as described by Doss (2008). Briefly, 2 g of *T. sericea* leaf filtrate was added to 5 ml of 10% ammonium hydroxide in a test tube. Then 1ml of concentrated sulphuric acid was added to the mixture. Presence of flavonoids was indicated by the development of a yellow colour.

3.2.2 Terpenoids

The presence of terpenoids in the *T. sericea* leaf extract was determined as described by Ayoola *et al*. (2008). Briefly, 0.5 g of the *T. sericea* leaf extract was added to 2ml chloroform in a test tube following which 3ml of concentrated sulphuric acid was added to the mixture. Development of a reddish brown colour in the interface layer indicated the presence of terpenoids.

3.2.3 Saponins

The presence of saponins in the T. sericea leaves was determined as described by Ayoola *et al.* (2008). Briefly, 1 ml of *T. sericea* leaf resultant filtrate was added to 10 ml distilled water in flask and shaken for 30 seconds. Frothing confirmed the presence of saponins.

3.2.4 Tannins

The presence of tannins in the *T. sericea* leaves was determined as described by Ayoola *et al*. (2008). Briefly, 0.5 mg of *T. sericea* extract was boiled in 5 ml of distilled water in a beaker. Ferric chloride was then added. Development of a dark purple black colour showed the presence of tannins.

3.2.5 Anthraquinones

The presence of anthraquinones in the *T. sericea* leaves was determined as described by Ayoola *et al*. (2008). Briefly 5 g of *T. sericea* extract was boiled in 10 ml 2M sulphuric acid. The mixture was then filtered; the resultant filtrate was added to 5 ml chloroform to form two layers. The chloroform layer was then carefully pipetted into a test tube containing 1ml of diluted ammonium hydroxide and colour changes were observed.

3.3 Brine shrimp toxicity assay

3.3.1 Hatching of nauplii

Cysts of *Artemia salina* were hatched at room temperature in saline water (2% NaCl) overnight. The saline was aerated by bubbling atmospheric air in the hatching container using a 60ml syringe connected to an infusion pump (Harvard Apparatus, MA, USA).

3.3.2 Brine shrimp assay

Fifty milligrams (50 mg) of the *T. sericea* aqueous leaf extracts were dissolved in 5ml dimethylsulfoxide and made to 100ml with 2.9% saline solution. The extract solution was serially diluted to concentrations of 5000, 500, 50, 5, 0.5 and 0.05 $\mu g.ml^{-1}$. The extract solutions (2ml) were transferred in duplicate to a 24-well plate. Saline solution (2.9%) was used as a negative control. Potassium dichromate, which is toxic to brine shrimp, was used as a positive control at concentrations of 3000, 300, 30 and 3 $\mu g.ml^{-1}$. The freshly hatched nauplii were placed in the wells (10 per well) followed by incubation at room temperature for 24 hours. The numbers of dead nauplii were then counted percentage mortality determined and the half maximal lethal concentration (LC_{50}) calculated.

3.4 Ethical clearance for use of animals and the study site

The study, conducted in the Central Animal Services (CAS) unit at the Faculty of Health Sciences, University of the Witwatersrand, was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand, Johannesburg, South Africa (AESC number: 2012/29/05). Tissue assays were done in appropriate laboratories of the School of Physiology, University of the Witwatersrand.

3.5 Animals: feeding and housing

Forty 21-day weanling male Wistar rat pups were used in the experiment. The commercial rat chow used in this study was bought from EPOL Animal Feed Manufacturers [Epol®, Centurion, South Africa (protein 170 $g.kg^{-1}$, fat 25 $g.kg^{-1}$, fibre 70 $g.kg^{-1}$, calcium 25 $g.kg^{-1}$, phosphorus 6 $g.kg^{-1}$ and total lysine 6.5 $g.kg^{-1}$)]. The pups had *ad libitum* access to a standard rat chow. Each pup was individually housed in a perspex cage with clean wood shavings for bedding. Bedding was changed once a week. Room temperature was maintained at 22±2°C with lights on between 7am to 7pm. The pups were given 3 days familiarisation period before the start of the experimental period.

3.6 Treatment doses and drug vehicle medium preparation

The dosage of fenofibrate used in the current study was similar to that used by Legendre *et al.* (2002). The low and high dose of the aqueous *T. sericea* leaf extracts were similar to those used by Njomen *et al.* (2009) and Rao and Nammi (2006). The 2 ml gelatine cubes used as vehicle mediums for *T. sericea* aqueous leaf extract and administration were prepared as described by Kamerman *et al.* (2004). Briefly, 8g of Sheridans, clear, unflavoured edible gelatine [Retailer Brands (Pty) Ltd, South Africa], 17 g brown sugar (Sunsweet® Huletts, South Africa) and 5ml Beefy Bovril (Bokomo Foods, South Africa) were added to 100ml of warm water and then the solution was allowed to cool down and form solid moulds. The Fructose solution was prepared by adding commercial fructose (Huletts®, Fructose Concentrated Sweetness, Low GI, South Africa) to a litre of warm water in a beaker. Food Colouring [Robertsons, Red Food colouring, Retailer Brands (Pty) Ltd, South Africa] was added to the fructose solution to help with identification.

3.7 Treatments and experimental design

Forty 21-day old male Wistar pups were randomly allocated to five treatment regimens. Each treatment regimen had 8 pups. The pups had *ad libitum* access to a standard rat chow. Group 1 pups were given plain gelatine cubes and plain drinking water (PC + PW), group 2: plain gelatine cubes and 12% fructose solution (PC + FS), group 3: gelatine cubes containing fenofibrate at a concentration calculated to deliver 100 $mg.kg^{-1}$ body mass per day and 12% fructose solution (FF + FS), group 4: gelatine cubes containing low dose of the *T. sericea* extract at a concentration calculated to deliver 100 $mg.kg^{-1}$ and 12% fructose solution (LD + FS) and

group 5: gelatine cubes containing high dose of the *T. sericea* extract at a concentration calculated to deliver 400 $mg.kg^{-1}$and 12% fructose solution (HD + FS). The pups were maintained on the treatment regimens for 12 weeks.

3.8. Body mass measurement

The rats were weighed (Snowrex Electronic Scale, Clover Scales, Johannesburg) twice per week to monitor growth performance. The body mass measurements also allowed for the maintenance of a constant dosage of the *T. sericea* leaf extract and fenofibrate relative to body mass over the 12-week treatment period.

3.9. Experimental intervention and terminal procedures

3.9.1 Oral glucose tolerance test

After 12 weeks of treatments the rats were given a 12-hour overnight fasting period before the determining their tolerance to an oral glucose challenge. Fasting blood glucose concentrations were determined (time interval 0) on each rat using a glucometer calibrated according to manufacturer' instructions (Ascentia, Elite^{TM}, Bayer Corporation, Mishawaka, USA). Blood for the fasting blood glucose concentration determination and after an oral glucose challenge was taken via a pin prick of the tail vein after sterilisation of the area to be pricked with a cotton gauze swab impregnated with alcohol (Loxham *et al.*, 2007). Following the determination of the

fasting blood glucose concentrations, rats were gavaged via orogastric intubation with 2g kg^{-1} body mass of sterile 50% (w/v) D-(+)-glucose solution [Merck Chemicals (Pty) Ltd, Johannesburg, South Africa]. Post gavage glucose concentrations at time intervals 15, 30, 60, 120 and 180 minutes, were then determined as previously described (Leng *et al.*, 2004).

3.9.2 Terminal procedures

Following the OGTT, the rats were put back onto their respective treatments for 48 hours. Thereafter, they were fasted overnight and their fasting blood glucose levels were then determined (the following morning), using a calibrated glucometer (Ascensia Elite^{TM} Blood glucose meter, Bayer Corporation, Mishawaka, USA) according to the manufacturer's instructions. The rats were then euthanased by intra-peritoneal injection with an overdose of sodium pentobarbitone (Eutha-naze, Bayer, Johannesburg, South Africa) at 200 $mg.kg^{-1}$ body mass.

3.9.2.1 Blood collection, processing and plasma storage

Following euthanasia of the rats, 8ml of blood was collected via cardiac puncture using 21G needles and 10ml syringes and divided equally into 4ml plain and 4ml heparin blood tubes (Becton Dickinson VACUTAINER Systems Europe, Meylan Cedex, France). A drop of the blood was then used to determine fasting blood triglycerides using a calibrated Accutrend triglyceride meter (Roche, Mannheim, Germany) according to the manufacturer's instructions. The blood samples were spun for 15 min at 5000 G at 20 ^{0}C in a Sorvall RT® 6000B centrifuge

(Pegasus Scientific Inc., Rockville USA). The plasma and serum were collected and then stored at -20 ^{0}C for the analysis of insulin and general health profile markers, respectively.

3.9.2.2 Determination of visceral organ morphometry

Following blood collection, the abdomen was cut open by midline incision. The liver, stomach, pancreas, small and large intestines, ceacum, visceral fat, epididymal fat, heart and kidney were carefully dissected out. The contents of the stomach, small and large intestines and caecum were gently emptied after which the GIT and other viscera were weighed on a Presica 310M electronic balance (Presica Instruments AG, Switzerland). The small and large intestines were gently stretched out on a board and their lengths were measured using a ruler mounted on a cooled dissection board. A sample of the liver was stored at -20ºC for determination of lipid and glycogen content. A sample of the liver and small intestine (proximal and distal) were preserved in 10% phosphate buffered formalin for histology analysis. The remainder of the liver samples were frozen for the determination of liver lipid and glycogen content. The masses of the empty carcasses were then determined after.

3.9.2.3 Determination of indexes of long bone growth

The femoral attachment of left hind leg to the pelvis was excised from each of the carcasses, defleshed and disarticulated from the tibia. The bones were dried in an oven (Salvis ®, Salvis Lab, Switzerland) at 50°C for 5 days and then weighed after which the tibia (measured between tibia head medial malleolus) and femur (measured between distal femoral articular surface to the

greater trochanter) lengths were measured with a pair of vernier calipers (Hi-impact, Dejuca, South Africa). Bone density was then caluclated using the formula:

Bone density = mass of bone (mg) /length of bone (mm)

To further evaluate bone density of tibiae and femora, radiographs were taken using a Fuji film X-ray machine (Industrial X-ray film FR, Fuji Photo Film Co, Ltd, Tokyo, Japan). Briefly, the bones were placed on the photographic plate at a distance of 1 metre from the X-ray light source with settings of 4.8 kVp, 0.71 mA per plate was used.

3.9.2.4 Determination of liver lipid and glycogen storage

Liver lipids were extracted as described by Bligh and Dyer (1959). Briefly, 5g of liver was steeped overnight in 150 ml of a 2:1 mixture of chloroform methanol at 4 ^{o}C. The mixture was filtered through filter paper (Whatmann®, No 1, size 185 mm, pore size 7-11µm, England) into 250ml separating funnel. Then 30ml of 0.9% saline was added to the filtrate and mixed thoroughly. The resultant mixture was allowed to stand overnight in a refrigerator at 4°C after which the organic layer was separated out into round bottomed flask. The organic aliquot was reduced to dryness under vacuum at 37°C. The lipid extract was dissolved in 20ml chloroform and 2 ml of the extract mixture was dried to determine the lipid content in the liver.

Liver glycogen was determined indirectly by acid hydrolysis to glucose and measurement of glucose (Passonneau and Lauderdale, 1974). The glucose concentration of the hydrosylate was determined on an Accu-Chek Active glucometer (Roche, Germany).

3.9.2.5 Determination of liver and small intestine histomorphometry

The preserved liver and small intestine samples were embedded in paraffin wax, sectioned, and then stained with haematoxylin and eosin, on a glass slide and covered with a glass cover slip (Reyes-Gordillo *et al.,* 2007). The slides were then viewed under a light microscope using an eye piece micrometer (Reichert®, Austria). The height of villi and crypt depth of the small intestines were measured. The hepatocyte size and cell numbers within a linear field (100μm) was measured at high power magnification of 400X.

3.9.2.6 Determination of biochemical health profile markers

Serum concentrations of ALT, ALP, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, phosphorus, calcium, cholesterol and amylase of the rats were determined using a colorimetric-based clinical chemistry analyzer (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions. Briefly, each stored serum sample was thawed and allowed to warm to room temperature, gently inverted to mix the contents and then placed into the analyzer which automatically drew up 150 μL of the serum. Ten microlitres (10 μL) of serum were then loaded onto each of the pre-loaded disks after which each sample was then analysed and print outs provided.

3.9.2.7 Plasma insulin concentration determination

Plasma insulin was determined by enzyme linked immunosorbent assay (ELISA) using a Rat insulin kit (DRG ®, Rat Insulin High Range, USA) according to the manufacturer's instructions. The assay employed a quantitative sandwich enzyme immunoassay technique which utilizes a monoclonal antibody specific for rat insulin. Absorbencies 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 concentration sand the concentrations of insulin in the samples were determined. A detailed description of the protocol employed is described in appendix 2.

3.9.2.8 Computation of the HOMA IR index

The HOMA-IR used for the assessment of insulin resistance, was computed as shown in the equation below:

HOMA-IR = [fasting insulin ($\mu U.ml^{-1}$) × fasting glucose ($mmol.l^{-1}$)/22.5] (Matthews *et al.*, 1985)

3.10 Statistical analysis

All data are expressed as mean ± SD. Data analysis was done using Graphpad Prism 5 software (Graph-pad Software Inc., San Diego, USA). Statistical significance was considered when $P \leq 0.05$. Data for weekly body mass and OGTT were analysed using a repeated measures analysis of variance while data for other parameters were analysed by a one-way analysis of variance. The

Bonferroni post hoc test was used to compare means. The model used for the analysis of variance for weekly body masses and the glucose tolerance test was:

$Y_{ijk} = \mu + T_i + B_j + C_k + e_{ijk}$; where;

Y_{ijk} = blood glucose concentration at time C post gavage

μ = overall mean common to all observations

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

B_j = fixed effect of individual rat (1,2,3.....40)

C_k = fixed effect of sampling time on blood glucose concentration (= 1,2.....6)

e_{ijk} = residual random error

The model used for analysis of variance for variables determined at study termination was:

$Y_{ijk} = \mu + T_i + B_j + e_{ijk}$; where;

Y_{ijk} = response variable of interest

μ = overall mean to all observations

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

B_j = fixed effect of individual rat (1,2,3.....40)

e_{ijk} = residual random error

CHAPTER 4: RESULTS

4.1 Phytochemical composition of aqueous leaf extract

Table 4.1 shows the phytochemical composition of the aqueous *T. sericea* leaf extract.

Table 4.1 Phytochemical constituents of the aqueous *T. sericea* leaf extract

Phytochemical	Present/Absent
Terpernoids	Present
Saponins	Present
Flavonoids	Present
Tannins	Present
Anthraquinones	Absent

The aqueous *T. sericea* leaf extracts contained terpernoids, saponins, flavonoids, and tannins but did not appear to contain anthraquinones.

4.2 Brine shrimp toxicity assay

Figure 4.1 shows the results of the toxicity test of aqueous *T. sericea* leaf extracts on brine shrimp nauplii.

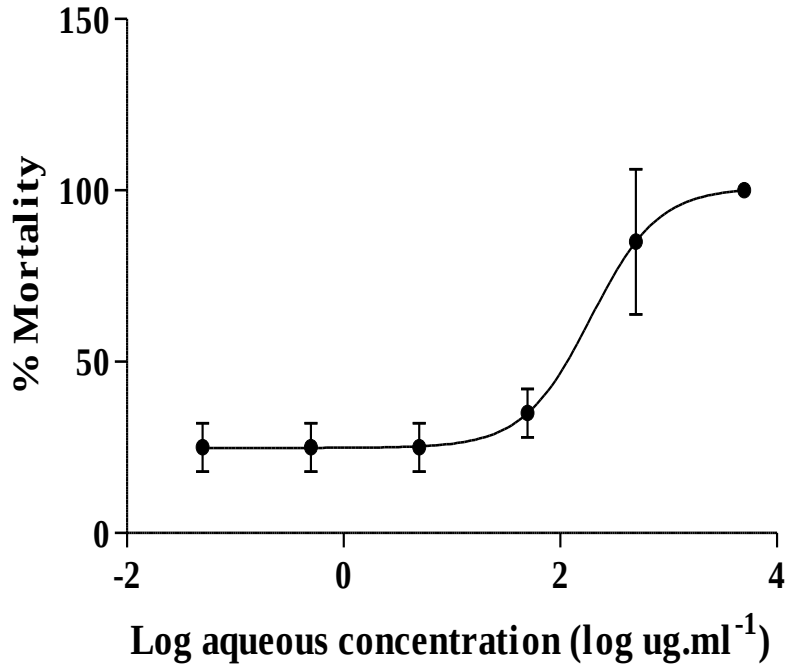

Figure 4.1: Toxicity of aqueous *T. sericea* extracts on *Artemia salina* (brine shrimp) nauplii.

The aqueous *T. sericea* leaf extracts had a median lethality concentration of (LC_{50} - 24 hours) of 97.96±0 µg.ml^{-1}.

While the median lethality concentration of (LC_{50} - 24 hours) of aqueous *T. sericea* leaf extracts was 97.96±0 $\mu g.ml^{-1}$ (Figure 4.1). The LC_{50} - 24 hours was 3 $\mu g.ml^{-1}$ for the positive control; potassium dichromate (data not shown).

4.3 Mortality and morbidity of the rats

During the *in vivo s*tudy, all the rats remained healthy with no incidental or iatrogenic rat mortalities recorded.

4.4 Effects of aqueous *T. sericea* leaf extract on growth performance

4.4.1 Body mass and empty carcass mass

Figures 4.2A and 4.2B show the induction and terminal body masses and empty carcass masses, respectively, of the rats across the different treatment regimens.

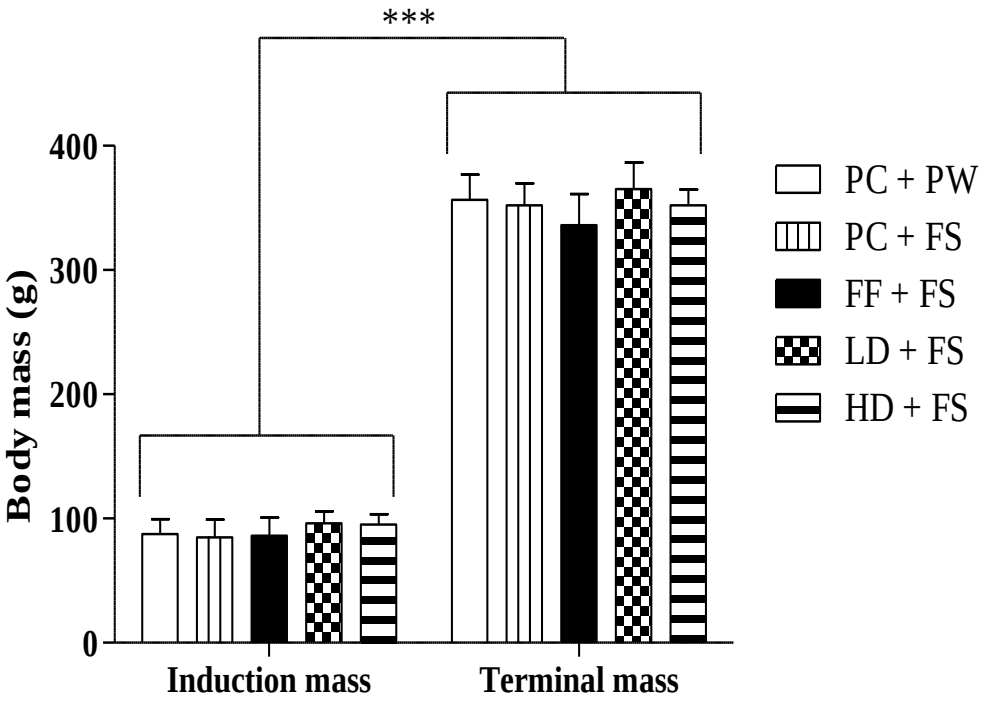

Figure 4.2A: The induction and terminal body masses of the rats given the different treatments.

*** $P < 0.0001$. The induction body masses of the rats were statistically similar ($P > 0.05$) across the different treatments. The terminal body masses of the rats were similar ($P > 0.05$) across the different treatments. Rats in all groups grew significantly ($P < 0.0001$) compared to their induction mass. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution. Data presented as mean±SD; n = 8 per treatment.

While the induction and terminal body masses of the rats across the treatments were similar ($P > 0.05$), rats given each treatment grew significantly ($P < 0.0001$) compared to their induction body masses (Figure 4.2A)

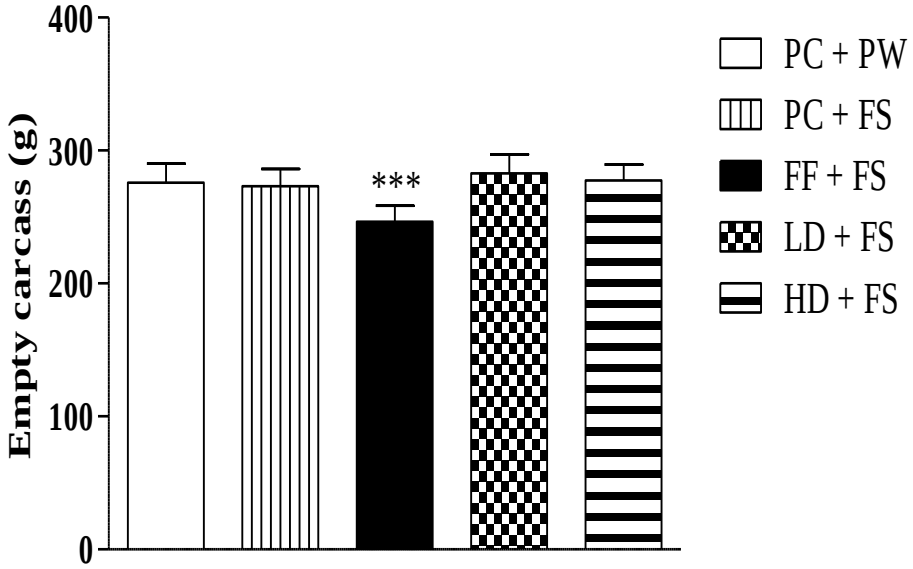

Figure 4.2B: Effect of aqueous *T. sericea* leaf extract on the empty carcass mass of the rats.

*** $P < 0.0001$. Rats given FF + FS had significantly ($P < 0.0001$) lighter empty carcass masses compared to empty carcass masses of rats from all treatment groups. Rats given PC + PW, PC + FS, LD + FS and HD + FS, respectively, had similar empty carcass mass. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution. Data presented as mean±SD; n = 8 per treatment.

While the empty carcass masses of rats given FF + FS were significantly ($P < 0.0001$) lower compared to the carcass masses of all rats from other treatment groups, rats given PC + PW, PC + FS, LD + FS and HD + FS, that had similar ($P > 0.05$) empty carcass masses (Figure 4.2B).

4.4.2 Linear growth

Table 4.2 shows lengths, masses and densities of tibiae and femora of the rats following administration of their respective treatments for 12 weeks.

Table 4.2: Effect of aqueous *T. sericea* leaf extract on tibiae and femora lengths, masses and density of the rats

	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
Tibia						
Length (mm)	40.74±0.66[a]	40.51±0.80[a]	39.65±0.71[b]	40.92±0.66[a]	40.56±0.65[a]	*
Mass (mg)	577.75±31.43[ab]	573.00±35.92[ab]	551.75±26.24[a]	598.5±28.08[b]	585.62±24.68[ab]	**
Density ($mg.mm^{-1}$)	14.18±0.61[a]	14.15±0.90[a]	13.91±0.57[a]	14.62±0.53[a]	14.43±0.53[a]	ns
Femur						
Length (mm)	36.45±0.97[a]	35.65±1.70[a]	35.48±0.93[a]	36.34±0.24[a]	37.01±1.55[a]	ns
Mass (mg)	676.00±36.21[a]	671.5±37.83[a]	653.3±48.26[a]	698.8±21.11[a]	686±32.52[a]	ns
Density ($mg.mm^{-1}$)	18.54±0.71[a]	18.86±1.08[a]	18.40±0.93[a]	19.23±0.55[a]	18.55±0.85[a]	ns

n.s. = not significant, $P > 0.05$. *$P \leq 0.05$; **$P < 0.01$. [ab]Within row means with different superscripts are significantly different at $P \leq 0.05$. Rats given FF + FS had significantly ($P = 0.04$) shorter tibiae compared to the tibiae lengths of rats from all treatment groups. Rats given LD + FS had significantly ($P = 0.0024$) heavier tibiae compared to that of rats given FF + FS. Femora lengths and tibiae and femora densities of the rats across the different treatments were similar ($P > 0.05$). PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution;

LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution. Data presented as mean±SD; n = 8 per treatment.

The rats that received FF + FS had significantly ($P = 0.04$) shorter tibiae compared to rats from all other treatment groups, which (PC + PW, PC + FS, LD + FS and HD + FS) had similar ($P > 0.05$) tibiae lengths (Table 4.2). While the rats given FF + FS had significantly ($P = 0.0024$) lighter tibiae compared to their counterparts given LD + FS, rats given other treatments (PC + PW, PC + FS and HD + FS) had similar ($P > 0.05$) tibiae masses (Table 4.2). Femora lengths and masses as well as tibiae and femora densities were similar ($P > 0.05$) across treatment groups (Table 4.2).

Figure 4.3 shows radiographs of femora and tibiae of the rats after 12 weeks on their respective treatments.

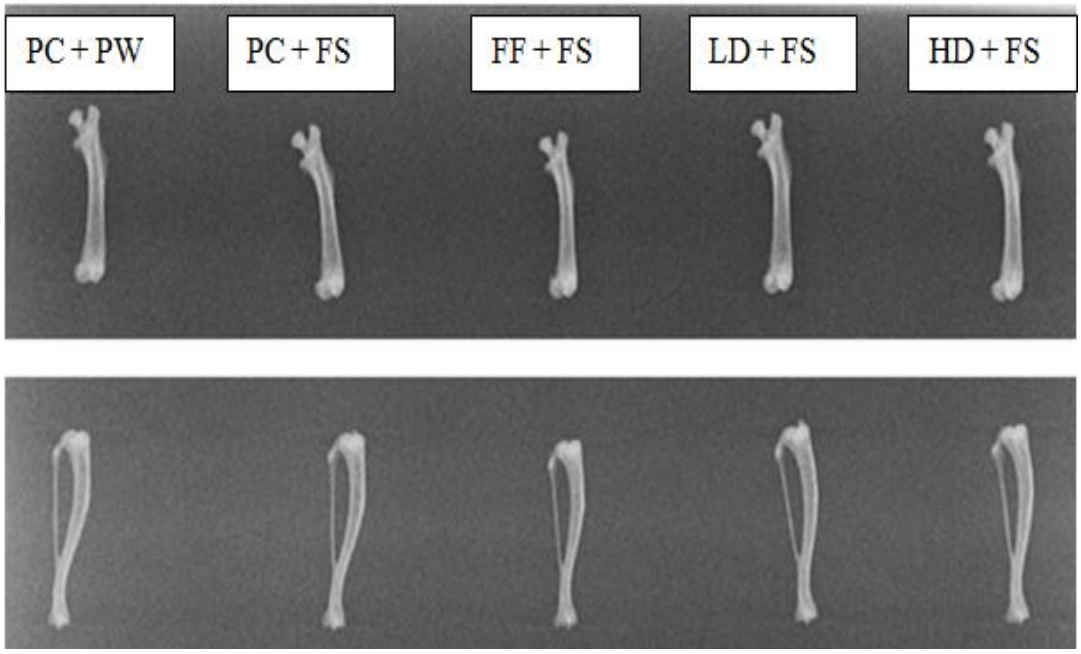

Figure 4.3: Radiograph images of femora and tibiae of the Wistar rats.

The top row shows radiographs of the femora of rats from different treatment regimens and the bottom row shows the tibiae of representative rats from the different treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 mg.kg $^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution.

There were no observable differences in the radiographical densities of the bones across treatment regimens (Figure 4.3).

4.5 Effect of aqueous *T. sericea* leaf extract on glucose tolerance

Figures 4.4A shows the blood glucose concentrations at basal (0 min) and at 15, 30, 60, 120 and 180 minutes post gavage with 50% percent glucose solution at 2 $g.kg^{-1}$ body mass of the rats following 12 weeks on their respective treatment regimens. Figure 4.4B shows the area under the curve calculated from oral glucose tolerance test results.

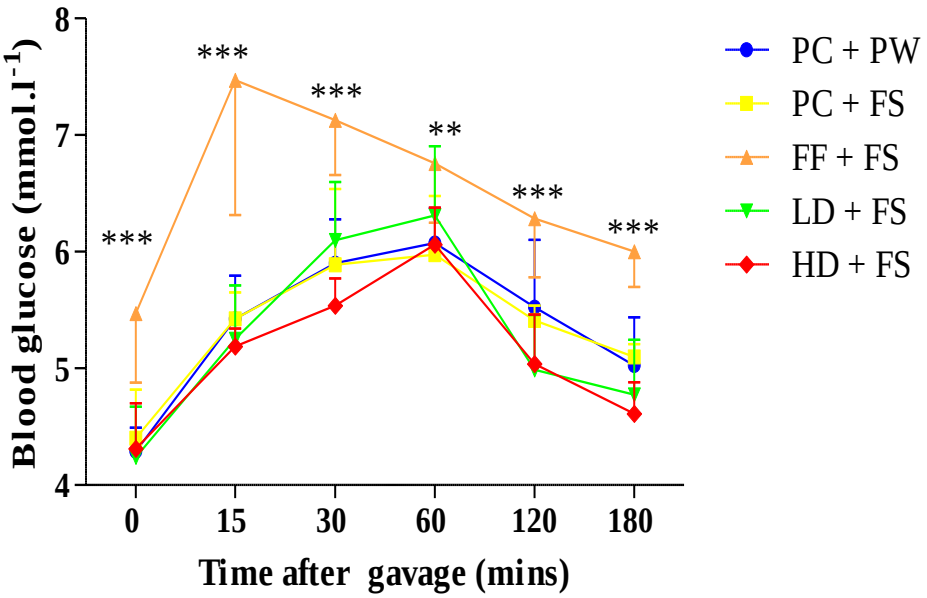

Figure 4.4A: Effect of aqueous *T. sericea* leaf extracts on the glucose tolerance of rats.

** P < 0.01; *** P < 0.0001. Rats given FF + FS 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 (rats given FF + FS) had significantly higher (P = 0.0006) blood glucose concentration compared to rats given PC + FS. The blood glucose concentration of rats given FF + FS significantly peaked (P < 0.0001) at 15 mins post-gavage while that of rats given other treatments significantly peaked (P < 0.0001) at 60 mins post gavage. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 mg.kg $^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution; Mins = minutes. Data presented as mean±SD; n = 8 per treatment.

The rats administered FF + FS had a significantly higher ($P < 0.0001$) basal blood glucose concentration and at 15, 30, 120, and 180 min post-gavage compared to rats given other treatments (Figure 4.4A). At 60 min post-gavage the rats that were given fructose solution and fenofibrate (FF + FS) had significantly ($P = 0.0006$) higher blood glucose concentration compared to their counterparts given plain cubes and fructose solution (PC + FS) (Figure 4.4A). While the blood glucose concentrations of rats given FF + FS returned to basal concentration 60 minutes post-gavage, those given LD + FS and HD + FS returned to basal 180 minutes post-gavage and those treated with PC + PW and PC + FS remained significantly higher ($P < 0.05$) compared to basal concentrations until 180 minutes post-gavage (Figure 4.4A).

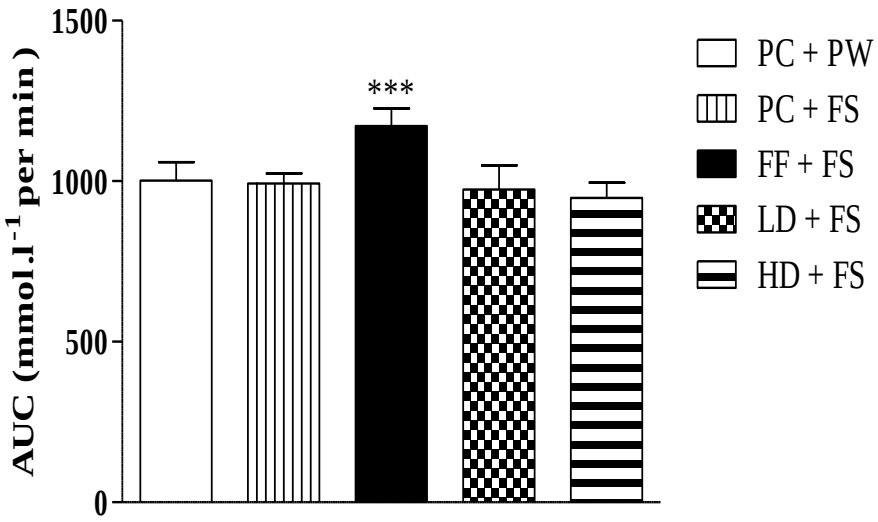

Figure 4.4B: Effect of aqueous *T. sericea* leaf extracts on total area under the curve of oral glucose tolerance of rats.

*** $P < 0.0001$. Rats treated with FF + FS had significantly ($P < 0.0001$) higher area under the curve of oral glucose tolerance test compared to that of rats from other treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 mg.kg $^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution; AUC = Area under curve. Data presented as mean±SD; n = 8 per treatment.

The area under the curve of oral glucose tolerance test of rats admintered FF + FS was significantly ($P < 0.0001$) higher compared to the area under the curve of oral glucose tolerance test from rats given other treatment regimens, which (rats from other treatment groups) had similar ($P > 0.05$) area under the curve of oral glucose tolerance test (Figure 4.4B).

4.6 Effect of aqueous *T. sericea* leaf extracts plasma insulin concentration and HOMA index

Table 4.3 shows the plasma insulin concentrations and the HOMA-IR indexes of the rats after being given their respective treatments for 12 weeks.

Table 4.3 Effect of aqueous *T. sericea* leaf extracts on fasting blood glucose and plasma insulin concentration and HOMA-IR index

	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
Glucose mmol.l^{-1}	4.14±0.36^{a}	4.01±0.44^{a}	5.14±0.54^{b}	4.06±0.35^{a}	3.95±0.36^{a}	***
Insulin µg.l^{-1}	5.65±2.80^{a}	5.32±2.28^{a}	5.93±2.21^{a}	5.40±1.73^{a}	5.21±1.50^{a}	ns
HOMA-IR	1.05±0.55^{a}	0.95±0.43^{a}	1.34±0.51^{a}	0.97±0.33^{a}	0.93±0.31^{a}	ns

n.s. = not significant, $P > 0.05$. ***$P < 0.0001$. ^{ab}Within row means with different superscripts are significantly different at $P \leq 0.05$. Rats given FF + FS had significantly higher ($P < 0.0001$) fasting glucose concentration compared to all rat treatment groups. The plasma insulin concentrations and HOMA-IR were statistically similar ($P > 0.05$) across for rats from all treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 mg.kg $^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution; HOMA-IR = Homeostatic model of insulin resistance. Data presented as mean±SD; n = 8 per treatment.

While rats adminitered FF + FS had significantly elevated ($P < 0.0001$) fasting blood glucose concentration compared to that of rats from the rest of the treatments, the plasma insulin concentration and the HOMA-IR index were similar ($P > 0.05$) for the rats across the treatment groups (Table 4.3).

4.7 Effect of aqueous *T. sericea* leaf extracts on visceral organs

The effect of the treatments on the absolute and relative masses and lengths (where appropriate) of the GIT viscera is shown in Table 4.4A while the masses of the other visceral organs are shown in Table 4.4B.

Table 4.4A: Effect of aqueous *T. sericea* leaf extract on GIT visceral organ masses and lengths in male Wistar rats

Organ	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
Stomach (g)	1.93±0.13^{a}	1.90±0.20^{a}	2.18±0.22^{a}	2.10±0.27^{a}	1.97±0.19^{a}	ns
%BM	0.54±0.03^{a}	0.54±0.06^{a}	0.64±0.04^{b}	0.57±0.05^{ab}	0.55±0.04^{a}	**
TLr (g.mm^{-1})	4.74±0.30^{a}	4.70±0.47^{a}	5.50±0.57^{b}	5.12±0.60^{ab}	4.84±0.44^{ab}	*
SI (mm)	1203±40.96^{a}	1243±102.4^{a}	1223±37.32^{a}	1172±89.17^{a}	1208±38.91^{a}	ns
SI (g)	7.41±0.71^{a}	7.27±0.48^{a}	8.38±1.03^{b}	7.60±0.60^{a}	7.31±0.41^{a}	*
%BM	2.07±0.14^{ab}	2.06±0.09^{ab}	2.47±0.15^{b}	2.08±0.08^{ab}	1.80±0.73^{a}	**
TLr (g.mm^{-1})	18.18±1.58^{a}	17.94±1.11^{a}	21.14±2.56^{b}	18.56±1.24^{a}	18.02±0.93^{a}	**
LI (mm)	248.10±20.34^{a}	268.80±14.33^{a}	261.30±14.58^{a}	254.40±10.16^{a}	260.00±18.52^{a}	ns
LI (g)	2.15±0.32^{a}	2.02±0.13^{a}	2.15±0.27^{a}	1.99±0.22^{a}	2.04±0.23^{a}	ns
%BM	0.60±0.07^{a}	0.57±0.04^{a}	0.64±0.09^{a}	0.54±0.06^{a}	0.57±0.06^{a}	ns
TLr (g.mm^{-1})	5.27±0.77^{a}	4.98±0.30^{a}	5.41±0.67^{a}	4.86±0.51^{a}	5.02±0.53^{a}	ns
Caecum (g)	1.48±0.24^{a}	1.38±0.25^{a}	1.22±0.18^{a}	1.33±0.24^{a}	1.29±0.24^{a}	ns

%BM	0.42±0.06[a]	0.39±0.05[a]	0.36±0.05[a]	0.36±0.06[a]	0.36±0.06[a]	ns
TLr (g.mm^{-1})	3.64±0.53[a]	3.40±0.62[a]	3.08±0.44[a]	1.33±0.24[b]	3.18±0.58[a]	***

n.s. = 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 adminitered FF + FS had significantly (P < 0.01) heavier (relative to body mass) stomachs compared to rats treated with PC + PW, PC + FS and HD + FS. Rats administered FF + FS had significantly (P < 0.05) heavier (relative to tibia length) stomachs compared to rats treated with PC + PW and PC + FS. Rats given FF + FS had significantly (P < 0.05) heavier absolute and relative (to tibia length) small intestine masses compared to all rat treatment groups. Rats given FF + FS had significantly (P = 0.002) heavier (relative to body mass) small intestine masses compared to rats given LD + FS. Rats administered the LD + FS had significantly (P < 0.0001) lighter caeca (relative to tibia length) compared to other treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 mg.kg $^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution; BM = Body mass; LI = Large intestine; SI = small intestine; TLr = Relative tibia length; VFP = visceral fat pad. Data presented as mean±SD; n = 8 per treatment.

Rats given FF + FS had significantly ($P < 0.01$) heavier stomachs relative to body mass compared to the stomach masses of rats given PC + PW, PC + FS and HD + FS (Table 4.4A). Relative to tibia length, rats administered FF + FS had significantly heavier stomachs ($P < 0.05$) compared to the stomach masses of rats treated with PC + PW and PC + FS (Table 4.4A). On the basis of absolute and relative to tibia length mass, rats administered FF + FS had significantly ($P < 0.05$) heavier small intestines compared to the small intestinal masses of rats administered other treatments (Table 4.4A). However relative to body mass the rats treated with FF + FS had significantly ($P = 0.002$) heavier small intestines compared to that of rats treated with LD + FS (Table 4.4A). Relative to tibia length, rats that were given FF + FS had significantly ($P < 0.05$) heavier caeca masses compared rats given other treatments (Table 4.4A). The large intestine absolute and relative (body mass and tibia length) masses and small intestine and large intestine lengths were similar ($P > 0.05$) across treatment groups (Table 4.4A).

Table 4.4B: Effect of aqueous *T. sericea* aqueous leaf extract on other visceral organs absolute and relative masses in male Wistar rats

Organs	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
Heart (g)	1.24±0.11^{a}	1.24±0.12^{a}	1.28±0.14^{a}	1.23±0.16^{a}	1.16±0.06^{a}	ns
%BM	0.35±0.02^{ab}	0.35±0.03^{ab}	0.38±0.04^{b}	0.35±0.03^{ab}	0.32±0.01^{a}	*
TLr (g.mm^{-1})	3.04±0.24^{a}	3.06±0.28^{a}	3.23±0.36^{a}	3.12±0.40^{a}	2.87±0.16^{a}	ns
Liver (g)	10.31±1.12^{a}	10.56±1.11^{a}	16.36±2.01^{b}	10.68±1.11^{a}	10.42±0.88^{a}	***
%BM	2.88±0.20^{a}	3.00±0.28^{a}	4.83±0.32^{b}	2.92±0.18^{a}	2.92±0.12^{a}	***
TLr (g.mm^{-1})	25.30±2.70^{a}	26.10±3.01^{a}	41.25±5.01^{b}	23.86±2.66^{a}	25.66±1.94	***
Pancreas (g)	1.58±0.28^{a}	1.47±0.24^{a}	1.59±0.30^{a}	1.60±0.33^{a}	1.55±0.17^{a}	ns
%BM	0.44±0.07^{a}	0.41±0.06^{a}	0.47±0.10^{a}	0.44±0.08^{a}	0.43±0.04^{a}	ns
TLr (g.mm^{-1})	3.88±0.67^{a}	3.62±0.61^{a}	4.00±0.75^{a}	3.92±0.83^{a}	3.83±0.40^{a}	ns
VFP (g)	5.89±1.57^{a}	6.66±1.10^{a}	7.51±1.63^{a}	7.23±2.32^{a}	6.68±1.27^{a}	ns
%BM	1.64±0.37^{a}	1.88±0.26^{a}	2.21±0.35^{a}	1.96±0.54^{a}	1.87±0.31^{a}	ns

EFP (g)	4.86±0.93[a]	4.97±0.84[a]	4.38±1.12[a]	5.46±0.91[a]	4.90±0.68[a]	ns
%BM	1.36±0.22[a]	1.41±0.23[a]	1.28±0.25[a]	1.50±0.18[a]	1.37±0.18[a]	ns
Kidney (g)	2.53±0.24[a]	2.44±0.12[a]	2.90±0.26[b]	2.51±0.24[a]	2.50±0.16[a]	***
%BM	0.71±0.04[a]	0.69±0.03[a]	0.86±0.03[b]	0.68±0.04[a]	0.70±0.04[a]	***
TLr ($g.mm^{-1}$)	6.22±056[a]	6.01±0.36[a]	7.30±0.60[b]	6.13±0.59[a]	6.17±0.38[a]	***

n.s. = not significant, $P > 0.05$. *$P \leq 0.05$, ***$P < 0.0001$. [ab]Within row means with different superscripts are significantly different at $P \leq 0.05$. Rats administered FF + FS had significantly ($P = 0.0132$) heavier relative (to body mass) hearts masses compared to the mass of the hearts of rats administered HD + FS. Rats given FF + FS had significantly ($P < 0.0001$) heavier livers (absolute and relative to body mass and tibia length) compared to the liver masses of rats given the other treatments. Rats administered FF + FS had significantly ($P < 0.0001$) heavier kidneys (absolute and relative to body mass and tibia length) compared to the kidney masses from rats administered other treatments. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; BM = Body mass; EFP: Epididymal fat pad; TLr = Relative tibia length. Data presented as mean±SD; n = 8 per treatment.

While absolute heart masses across all treatment groups was similar ($P > 0.05$), relative to body mass rats administered FF + FS had significantly ($P = 0.0132$) heavier hearts compared to the heart masses of rats administered HD + FS (Table 4.4B). Relative to body mass and tibia length, rats that were administered FF + FS had significantly ($P < 0.0001$) heavier kidneys and livers, respectively, compared to the kidney and liver masses from rats received other treatments (Table 4.4B). The visceral fat pad, epididymal fat pad, pancreas and testicular masses were similar ($P > 0.05$) across all treatment groups (Table 4.4B).

Table 4.4C shows the villi height, crypt depth and ratio of the villi heights to crypt depths of the rats given the different treatments. The mean hepatocyte size and number of hepatocytes per 100µm linear field is shown in Table 4.4D.

Table 4.4C: Effect of aqueous *T. sericea* aqueous leaf extract on villi height, crypt depth and the villi height: crypt depth ratio in male Wistar rats

	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
VH (µm)	51.83±2.42[a]	65.00±5.77[a]	66.12±7.95[a]	62.5±3.53[a]	58.5±9.19[a]	ns
CD (µm)	18.75±2.16[a]	19.50±1.00[a]	17.25±0.35[a]	17.75±4.95[a]	19.00±0.35[a]	ns
VH/CD ratio	2.76±1.12[a]	3.30±5.71[a]	3.83±0.54[a]	3.52±0.71[a]	3.08±26.26[a]	ns

n.s. = not significant, $P > 0.05$. [ab]Within row means with different superscripts are significantly different at $P \leq 0.05$. Villus height, crypt depth and the villus height: crypt depth ratios of the rats were similar ($P > 0.05$) across treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; VH = Villus height; CD = Crypt depth. Data presented as mean±SD; n = 8 per treatment.

Table 4.4D: Effect of aqueous *T. sericea* leaf extract on hepatocyte size and density of male Wistar rats

Liver	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
Hepatocyte size (µm)	9.37±0.90[a]	9.22±0.41[a]	9.81±0.54[a]	9.53±0.55[a]	9.67±0.84[a]	ns
Hepatocytes (cells per 100 µm)	10.53±0.90[a]	10.81±1.31[a]	10.41±0.48[a]	11.59±1.63[a]	12.13±1.46[a]	ns

n.s. = not significant, $P > 0.05$. [ab]Within row means with different superscripts are significantly different at $P \leq 0.05$. The number of intact hepatocytes in a linear field (100 µm) and hepatocyte size were statistically similar ($P > 0.05$) across all rat treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution. Data presented as mean±SD; n = 8 per treatment.

The villi height, crypt depth, villi height:crypt depth ratio, hepatocyte cell size and numbers of hepatocytes in a 100µm linear field were similar ($P > 0.05$) across all treatment groups (Tables 4.4C and 4.4D).

4.8 Effect of *T. sericea* aqueous leaf extracts on hepatic metabolic substrates

Figures 4.5A and 4.5B show liver glycogen (represented as glucose equivalents in the liver homogenate) and lipid content respectively, of the rats after being given their respective treatments for 12 weeks.

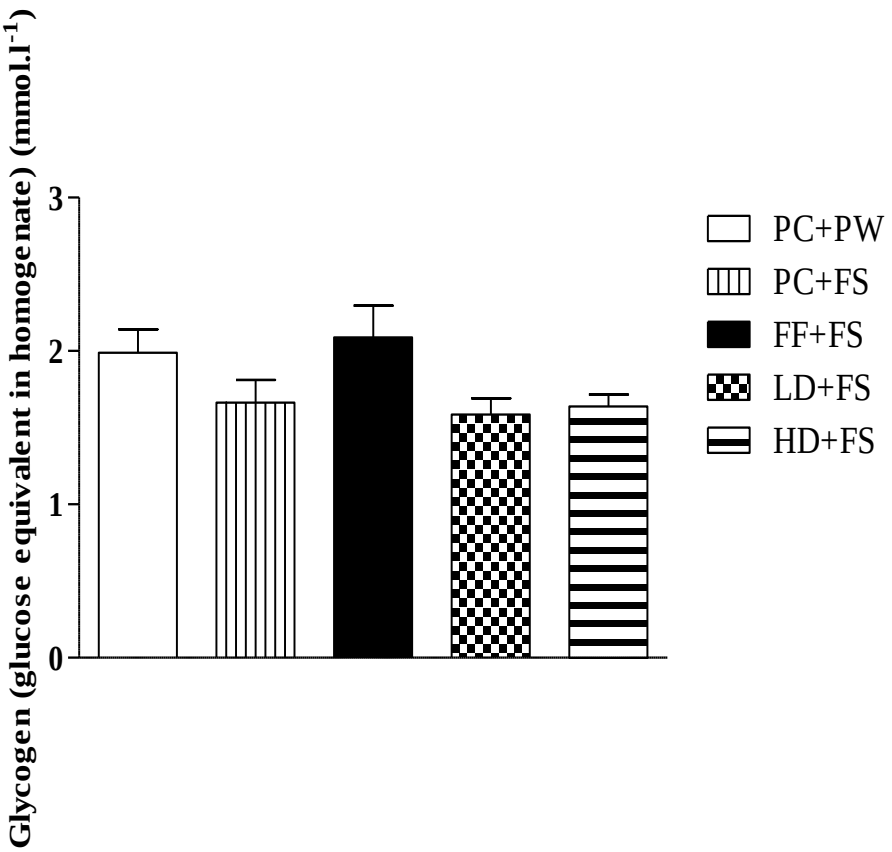

Figure 4.5A: Effect of aqueous *T. sericea* leaf extracts on the hepatic glycogen content of male Wistar rats.

Hepatic glycogen (represented as glucose equivalents in the liver homogenate) contents was statistically similar ($P > 0.05$) in the rats across the different treatment groups. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 mg.kg $^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 mg.kg^{-1} body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 mg.kg^{-1} body mass per day) + 12% fructose solution. Data presented as mean±SD; n = 8 per treatment.

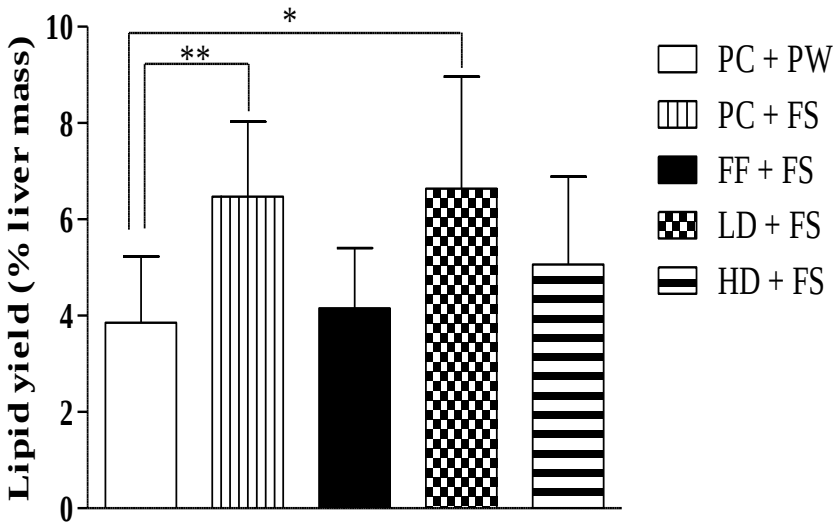

Figure 4.5B: Effect of aqueous *T. sericea* leaf extracts on the hepatic lipid content of male Wistar rats.

***** $P \leq 0.05$; ** $P < 0.01$. Rats given PC + FS and those given LD + FS had significantly ($P = 0.0032$; $P = 0.0114$, respectively) higher lipid yield (percentage liver mass) compared to rats given PC + PW (control). PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution. Data presented as mean±SD; n = 8 per treatment.

While the liver glycogen (represented as glucose equivalents in the liver homogenate) content of the rats across treatments were similar ($P > 0.05$; Figure 4.6B), rats that were given the plain gelatine cubes + 12% fructose solution (PC + FS) and their counterparts on a low dose of the aqueous *T. sericea* leaf extract + 12% fructose solution (LD + FS) had significantly higher ($P = 0.0032$; $P = 0.0114$, respectively) hepatic lipid content compared to rats that were given PC + PW (control) (Figure 4.6B).

4.9 Effect of aqueous *T. sericea* leaf extract on general health profile

Table 4.5 shows the effects on the markers of the general health of the rats following a 12 week administration of their respective treatments.

Table 4.5 The effect of aqueous *T. sericea* leaf extracts on serum markers of general health in male Wistar rats

Parameter	PC + PW	PC + FS	FF + FS	LD + FS	HD + FS	Significance level
ALT ($U.L^{-1}$)	64.13 ± 10.96^{a}	70.25 ± 18.61^{a}	80 ± 20.80^{a}	69.13 ± 10.60^{a}	70.25 ± 21.31^{a}	ns
ALP ($U.L^{-1}$)	112.6 ± 23.65^{a}	109 ± 19.12^{a}	347.40 ± 103.6^{b}	101.4 ± 19.26^{a}	102 ± 10.70^{a}	***
TBIL ($mg.dL^{-1}$)	0.4 ± 0.10^{a}	0.42 ± 0.14^{a}	0.46 ± 0.13^{a}	0.46 ± 0.12^{a}	0.38 ± 0.08^{a}	ns
Globulin ($g.dL^{-1}$)	2.01 ± 0.45^{a}	1.99 ± 0.64^{a}	1.71 ± 0.80^{a}	2.09 ± 0.55^{a}	1.76 ± 0.50^{a}	ns
Albumin ($g.dL^{-1}$)	3.41 ± 0.54^{a}	3.65 ± 0.62^{a}	4.05 ± 0.76^{a}	3.82 ± 0.44^{a}	3.82 ± 0.42^{a}	ns
Total protein ($g.dL^{-1}$)	5.45 ± 0.45^{a}	5.62 ± 0.54^{a}	5.77 ± 0.76^{a}	5.91 ± 0.40^{a}	5.57 ± 0.45^{a}	ns
BUN ($mg.dL^{-1}$)	19.88 ± 2.85^{a}	22.50 ± 2.78^{a}	23.88 ± 1.55^{a}	23.13 ± 2.36^{a}	23.25 ± 3.65^{a}	ns
Creatinine ($mg.dL^{-1}$)	0.40 ± 0.07^{a}	0.40 ± 0.12^{a}	0.38 ± 0.19^{a}	0.40 ± 0.13^{a}	0.45 ± 0.09^{a}	ns
Calcium ($mg.dL^{-1}$)	10.64 ± 0.94^{a}	10.88 ± 1.13^{a}	11.19 ± 2.04^{a}	11.06 ± 1.20^{a}	11.01 ± 0.82^{a}	ns
Phosphorus ($mg.dL^{-1}$)	5.46 ± 0.87^{a}	5.74 ± 0.51^{ab}	6.82 ± 1.16^{b}	6.22 ± 0.83^{ab}	5.54 ± 0.95^{ab}	*

Continued

Amylase ($U.L^{-1}$)	1349±271.9[a]	1522±232[a]	1588±306.4[a]	1495±114.2[a]	1424±51.98[a]	ns
Triglycerides ($mmol.l^{-1}$)	1.23±0.35[a]	1.53±0.37[a]	1.29±0.30[a]	1.38±0.27[a]	1.53±0.29[a]	ns
Cholesterol ($mg.dL^{-1}$)	69.75±10.74[a]	75.38±14.70[a]	75.38±31.30[a]	74.38±17.44[a]	74.25±10.47[a]	ns

* $P \leq 0.05$; *$P < 0.05$; ***$P < 0.0001$. [ab]Within row means with different superscripts are significantly different at $P < 0.05$. Rats administered FF + FS had significantly ($P < 0.0001$) higher serum ALP concentration compared to the serum ALP in rats given other treatments. Rats adminitered FF + FS had significantly ($P = 0.0346$) higher serum phosphate concentrations compared to that of rats given PC + PW. PC + PW = plain gelatine cube + plain water; PC + FS = plain gelatine cube + 12% fructose solution; FF + FS = 100 $mg.kg^{-1}$ body mass per day fenofibrate + 12% fructose solution; LD + FS = low dose aqueous *T. sericea* leaf extract (100 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; HD + FS = high dose aqueous *T. sericea* leaf extract (400 $mg.kg^{-1}$ body mass per day) + 12% fructose solution; ALP = Alanine phosphatase ALT = Alanine aminotransferase; BUN = Blood urea nitrogen; TBIL = Total bilirubin. Data presented as mean±SD; n = 8 per treatment.

Rats that were given FF + FS had significantly ($P < 0.0001$) elevated serum ALP concentrations compared to the serum ALP concentrations of rats from other treatment groups (Table 4.5). The serum phosphate concentration from rats given FF + FS was significantly ($P = 0.0346$) elevated compared to that from rats given PC + PW (Table 4.5). Other serum markers of the general health profile were similar ($P > 0.05$) across the treatments (Table 4.5).

CHAPTER 5: DISCUSSION

The discussion focuses on the phytochemical composition of aqueous *T. sericea* leaf extracts, *in vitro* toxicity of the aqueous *T. sericea* leaf extract, effects of the leaf extracts on the growth performance, glucose tolerance and HOMA-IR index, GIT viscera and other visceral organs, hepatic metabolite storage and the general health profile of growing male Wistar rats allowed to drink 12% fructose solution *ad libitum*.

5.1 Phytochemical constituents

Bessong *et al.* (2004) reported the presence of triterpene saponin and tannins in organic *T. sericea* leaf extracts while Nkobole *et al.* (2011) noted organic stem bark *T. sericea* extracts to contain triterpene saponin, tannins and flavonoids. Although this study investigated, qualitatively, the phytochemical composition of the aqueous *T. sericea* extract, results of the current study are in agreement with the findings of Nkobole *et al.* (2011) and Bessong *et al.* (2004). Triterpene saponins, tannins and flavonoids, which were reported present in the aqueous leaf extract of the current study, are hypothesised to give *T. sericea* extracts antioxidant, lipolytic an antidiabetic properties (Subramaniam *et al.*, 2011; Singh *et al.*, 2008: Tapas *et al.*, 2008). The presence of the phytochemicals (triterpene saponins, tannins and flavonoids) in the *T. sericea* extracts points to potential antioxidant, lipolytic and hypoglycaemic properties.

5.2 *In vitro* toxicity on Brine shrimp nauplii

Our Animal Ethics Screening Committee (AESC) does not permit the use of animals in toxicity studies, therefore the Brine shrimp toxicity assay served as a proxy to screen the potential toxicity of the aqueous *T. sericea* extracts before use in rats. In the current study aqueous *T. sericea* aqueous leaf extracts were not toxic to brine shrimp nauplii with LC_{50} of (97.96 µg.ml^{-1} after 24 hours (Figure 4.1), which is above the 20 µg.ml^{-1} cytotoxic cut off point stated by Moshi and Mbwambo (2005). Moshi and Mbwambo (2005) reported that aqueous *T. sericea* root extracts were highly toxic to brine shrimp nauplii with LC_{50} of 5.4 µg.ml^{-1}. Findings of the current study on low toxicity to brine shrimp of aqueous *T. sericea* leaf extracts are corroborated in the findings of the general health profile of the rats, as will be discussed later in this dissertation. Futhermore, these findings suggest that aqueous *T. sericea* leaf extracts could be used in ethnomedicine with a reduced possibility of them causing toxicity.

5.3 Growth performance

5.3.1 Body mass

Body mass can be affected by hydration state, GIT content fill and visceral organ size (Owens *et al.*, 1995), thus making it an inaccurate indicator of growth performance. Empty carcass mass is a good indicator of growth (lean) performance (Owens *et al.*, 1995). In the current study the administration of PC + PW, PC + FS, LD + FS and LD + FS had no effect on empty carcass masses. However the administration of FS+FS caused a decrease in empty carcass mass of the rats. These findings suggest that while the administration of fenofibrate to rats fed 12% fructose solution caused a reduction in the empty carcass mass,

while administration of both a low and high dose aqueous *T. sericea* leaf extracts to rats fed a 12% fructose solution had no negative effect on the empty carcass mass.

5.3.2 Linear growth

Long bones grow in response to growth hormone in a dose-dependent manner (Rol De Lama *et al*., 2000), thus making them a better proxy for the estimation of growth performance (Sanchez and He, 2009; Han *et al.*, 1965; Butler *et al.*, 1956). The rats that were given FF + FS had significantly shorter tibiae compared to the tibiae lengths from rats given other treatment regimens (Table 4.2). Additionally they had lighter tibiae compared to their counterparts given LD + FS (Table 4.2). These findings suggest that the administration of 100 $mg.kg^{-1}$ body mass per day of fenofibrate to rats fed a 12% fructose solution might have adverse effects on lengths of tibia. However the similarity in tibiae and femora densities (as determined by computation and by radiography), from rats across treatments (Table 4.2 and Figure 4.3) suggest that the effects of fenofibrate on linear growth need further investigation.

5.4 Glucose tolerance, HOMA-IR and plasma insulin

Previous studies have reported that in rats, high fructose diets cause hyperglycemia, glucose intolerance, hypertriglyceridaemia, hypercholesterolaemia and insulin resistance (Atanasovska *et al.*, 2009; Huang *et al.*, 2004). The similarities in the fasting blood glucose concentrations (Table 4.3; Figure 4.4A), glucose handling after an oral glucose challenge

(Figure 4.4A and 4.4B), area under curve of oral glucose tolerance test, plasma insulin concentration, HOMA-IR (Table 4.3), fasting blood triglyceride and cholesterol (Table 4.5) of rats given the control (PC + PW), PC + FS, LD + FS and HD + FS indicates that a 12% fructose solution administered for 12 weeks to growing male Wistar rats did not cause hyperglycaemia, impaired glucose handling, hypertriglyceridaemia, hypercholesterolaemia and insulin resistance. The current study results are at variance with the reported hyperglycaemia, hypertriglyceridaemia and insulin resistance reported in adult Wistar rats after administration of a 10% fructose solution for 8 weeks (de Moura *et al.,* 2009). However, in a study on the effect of age on markers of metabolic syndrome in Wistar rats, Ghezzi *et al.* (2012) reported that at ages 2 to 6 months the rats had lower serum concentrations of glucose, triglycerides, total cholesterol, HDL and LDL compared to their 12-month old counterparts. At study termination the rats in the current study were 3.75-months old, thus were still in the age group where the rats were reported to have lower serum markers of metabolic syndrome (Ghezzi *et al.,* 2012). The failure to develop signs (glucose intolerance, hypertriglyceridaemia, insulin resistance and hypercholesterolaemia) of metabolic syndrome by rats in the current study after being administered with a 12% fructose solution for 12 weeks could be attributed to their age. de Moura *et al.* (2009) hypothesised that young Wistar rats may have protective mechanisms against fructose-induced metabolic syndrome however, they did not provide insight into the specific mechanisms that could be responsible. Further investigations are thus required in order to establish the underlying mechanisms.

It was observed in the current study that the administration of fenofibrate for 12 weeks at 100 $mg.kg^{-1}$ body mass per day to growing Wistar rats fed a 12% fructose solution resulted in decreased glycaemic control as evidenced by significantly higher fasting blood glucose

concentration (Table 4.3) and failure to effectively control blood glucose concentrations following an oral glucose load (Figure 4.4A and 4.4B) by rats that were given FF + FS. Liu *et al.* (2011) reported that the administration of 100 $mg.kg^{-1}$ body mass per day of fenofibrate for 12 weeks in monosodium glutamate-induced obese rats decreased glucose stimulated insulin release and worsened their ability to handle glucose. While fenofibrate is reported to cause inflammation and oxidative stress to the pancreas which results in decreased insulin secretion (Liu *et al.,* 2011) and decreased ability to handle glucose, the decreased glycaemic control and hyperglycaemia in the rats given FF + FS suggests that the administration of the fenofibrate may have affected pancreatic β-cell function.

The administration of 100 $mg.kg^{-1}$ fenofibrate for 9 days to 8-10 week old male Wistar rats with hypertriglyceridaemia reduced plasma triglyceride concentration (Ferreira *et al.,* 2008). In the current study, the administration of fenofibrate at a similar dose for 12 weeks to rats fed a 12% fructose had no effect on blood triglyceride concentration. A study by Furuhashi *et al.* (2002) showed that fenofibrate (30 $mg.kg^{-1}$) administration for 6 weeks to 12-week old Sprague-Dawley rats with insulin resistance improved peripheral insulin sensitivity; in the present study similarity in the HOMA-IR index of the rats given fenofibrate and that of their counterparts on other treatments suggested that fenofibrate had no effect on insulin sensitivity.

In the present study, the low and high dose of aqueous *T. sericea* leaf extracts had no effect on the fasting blood glucose, triglyceride, insulin and glucose handling of growing male Wistar rats. Tannins, saponins and flavonoids in *Terminalia* species extracts have been shown to reduce fasting blood glucose concentrations, improve glucose handling, insulin

sensitivity and β-cell function in streptozotocin-induced diabetic rats (Latha and Daisy 2010; Tapas *et al.,* 2008; Murali *et al.,* 2007; Nagappa *et al.,* 2003; Francis *et al.,* 2002). *In vitro* studies by Nkobole *et al.* (2011) and Tshikalange *et al.* (2008) attributed the antidiabetic properties of *T. sericea* to the bioactive constituients- tannins, saponins and flavanoids found in it. Findings of the current study, (fasting blood glucose, glucose tolerance test and HOMA-IR indexes) suggest that despite the presence of tannins, saponins and flavonoids in aqueous *T. sericea* leaf extracts, administration of both a low and high dose of the leaf extracts had no effect on fasting blood glucose and triglyceride concentration and insulin sensitivity by peripheral tissue.

5.5 Visceral organs

5.5.1 GIT visceral organs

Nutrients are mainly absorbed in the small intestine. An increased villi height:crypt depth ratio indicates increased absorptive ability (Metges, 2010). The development of the different parts of the GIT can be affected by dietary consumption (Metges, 2010). Fenofibrate administration to 3-month old PPARα null mice at dose 800 $mg.kg^{-1}$ in feed for 10 days has been shown to alter gene expression in the intestine (Valasek *et al.,* 2007) which translated to reduced lipid absorption (Uchida *et al.,* 2011; Valasek *et al.,* 2007) and increased height of villi in the small intestine (Uchida *et al.,* 2011; Valasek *et al.,* 2007). In the current study, the oral administration of fenofibrate (100 $mg.kg^{-1}$ body mass per day) for 12 weeks to rats fed a 12% fructose solution resulted in increased absolute and relative (to body mass and tibia length) small intestine mass (Table 4.4A) but had no effect on villus height and crypt depth (Table 4.4C). The effects of fenofibrate on the small intestine

could have been due to altered digestion and absorption resulting in an accumulation of nutrients the intestine, which might (nutrient accumulation), have resulted in stretching of the small intestine tissues and thus their increased masses (Tormo *et al*., 2004; Younes *et al.*, 2001). However, the mechanism by which fenofibrate increased the small intestine mass of growing rats given 12% fructose solution requires further investigation. The current study reports that whilst administration of fenofibrate (100 $mg.kg^{-1}$ body mass per day) resulted in increased relative (to body mass) stomach mass; administration of low dose (100 $mg.kg^{-1}$ body mass per day) of aqueous *T. sericea* extract resulted in increased relative (to tibia length) caeca mass in Wistar rats fed 12% fructose solution. However these finding could have been due to the differences in body mass and tibia length that the relative values were calculated from. Tannins have been reported to erode the GIT mucosal layer (Chung *et al.*, 1998) and thus interfere with the digestive and absorptive function of the GIT. In the current study, the administration of aqueous *T. sericea* leaf extracts that contained tannins for 12 weeks had no negative effect on growth performance (body mass and linear), suggesting that GIT's digestive and absorptive function was not compromised hence nutrients were availed to support growth.

5.5.2 Other viscera

High fructose diets are known to have the potential to cause cardiovascular and renal system complications, such as microvascular and muscular tissue hypertrophy (Dachani *et al.*, 2012; Gersch *et al.*, 2007; Sánchez-Lozada *et al.*, 2007). Furthermore, they are known to increase liver mass and visceral fat pad mass (London and Castonguay, 2011; Huang *et al.*, 2004). In the current study following the administration of PC + FS to rats for 12

weeks, no changes in absolute and relative masses of the heart, kidney, visceral fat pad, epididymal fat pad, liver and pancreas were observed in growing Wistar rats. However, following the administration of FF + FS for 12 weeks, results of the current study point to increased heart relative (to body mass) masses in the growing Wistar rats (Table 4.4B) which are suggestive of increased cardiac tissue. Similar results were reported by Duhaney *et al.* (2007) where fenofibrate administration was found to result in increased ventricular hypertrophy in wild-type FVB mice. Thus, findings from the current suggest that the oral administration of fenofibrate might have adverse effect on the heart mass of Wistar rats given a standard rat chow but given a 12% fructose solution. However, since there were no statistically significant differences absolute masses of the hearts from the rats across treatments, this finding requires further investigation.

Fenofibrate has been shown to be reno-protective in metabolic syndrome models using adult mice and rats (Tanaka *et al.*, 2011; Park *et al.*, 2006). Results of the current study revealed that administration of fenofibrate ($100mg.kg^{-1}$ body mass per day) for 12 weeks to growing Wistar rats fed a 12% fructose solution, caused a significant ($P < 0.05$) increase in absolute and relative (to body mass and tibia length), in the masses of their kidneys (Table 4.4B). Kidney hypertrophy could be a result of microvascular infiltration of the kidney tissue which has potential to cause kidney disease, thus results of the current study seem to contradict the reported reno-protecitve effects of fenofibrate (Tanaka *et al.*, 2011; Park *et al.*, 2006).

Price *et al.* (1986) reported that fenofibrate can result in hepatomegally within 3 days of administration in rats. Hong *et al*. (2007) reported that the administration of fenofibrate

at100mg.kg^{-1} body mass per day for 6 weeks to adult male Sprague-Dawley rats resulted in hepatomegally. Clinical studies have demonstrated that fenofibrate causes inflammation of the liver which in turn leads to hepatomegally (Tolman, 2000). In the current study, the administration of fenofibrate (100mg.kg^{-1} body mass per day) for 12 weeks to growing male Wistar fed a 12% fructose solution caused a significant increase in absolute and relative (to body mass and tibia length) liver mass which might be indicative of hepatomegally (Table 4.4B), but without affecting hepatocyte size and the number of hepatocytes in a linear field (Table 4.4D). Results the current study are in agreement with the finding reported by Gasa (2012), Hong *et al.* (2007), Harano *et al.* (2006) and Price *et al.* (1986) also reported that fenofibrate causes hepatomegally.

The tannins, saponins and flavonoids of *Terminalia arjuna* and *Terminalia chebula* have been shown to be protective to the heart, kidney, liver and pancreas in alloxan-induced diabetic rats, adult male Wistar, NZW albino and Swiss albino rats (Muhammad *et al.*, 2012; Raghavan and Kumari, 2008; Sinha *et al.*, 2008; Manna *et al.*, 2006; Gauthaman *et al.*, 2005; Suchalatha and Shyamala, 2004; Gauthaman *et al.*, 2001; Dwivedi and Agarwal, 1994). In the current study following administration of aqueous *T. sericea* leaf extracts at low (100 mg.kg^{-1} body mass per day) and high (400 mg.kg^{-1} body mass per day) doses for 12 weeks to growing Wistar rats fed a 12% fructose solution, no changes in absolute and relative masses of the heart, kidney, visceral fat pad, epididymal fat pad, liver and pancreas were observed. These finding suggests that aqueous *T. sericea* leaf extracts might have no adverse effects on these viscera.

5.6 Liver metabolic substrate storage and markers of general health

The liver has several functions including: metabolite storage, bile production, protein synthesis, and inactivation of chemical messengers and detoxification (Govind, 2011; Salie *et al.,* 1991). High fructose diets are known to cause liver diseases by altering the metabolism of carbohydrates and lipids that leads to derangement of homeostasis (Rutledge and Adeli 2007; Thirunavukkarasu *et al.,* 2004). Conventional drugs and ethnomedicines may contain contaminants and toxins which are processed and detoxified in the liver and may have the potential to negatively influence liver function (Basha *et al.,* 2012; Hajdu *et al.*, 2009; Tasduq *et al.*, 2006; Manna *et al.,* 2006). It is therefore important to measure surrogate markers of liver function when ethnomedicines are used.

5.6.1 Liver glycogen content

Feeding a high-fructose diet to Wistar rats has been reported to reduce glycogen synthesis in the liver through reduced sensitivity to insulin by hepatocytes, which translates to decreased glucose uptake by the cells (Bezerra *et al.,* 2001). Decreased glucose uptake by hepatocytes results in reduced glycogen synthesis (Kumar *et al.,* 2006). Yadav *et al.* (2007) reported that administering 21% fructose solution for 8 weeks to 8-week old Wistar rats significantly increased their liver glycogen content. In the current study following administration of respective treatments for 12 weeks, no differences were observed in the liver glycogen content of rats given to the control (PC + PW), PC + FS and those on the low (LD + FS) and high (HD + FS) dose aqueous *T. sericea* leaf extract and those given fenofibrate (FF + FS). These findings suggest that the 12% fructose solution, fenofibrate

(100 mg.kg^{-1} body mass per day), and low (100 mg.kg^{-1} body mass per day) and high (400 mg.kg^{-1} body mass per day) aqueous *T. sericea* extracts did not affect fasting liver glycogen content of Wistar rat. Fasting is known to cause liver glycogen depletion (Gutman and Shafrir, 1964). The similarity in the liver glycogen content from the rats across treatments could have been due to the depletion of the liver glycogen stores, since the rats were fasted overnight prior to euthanasia. Future studies should consider investigating glycogen stores of rats in the fed state.

5.6.2 Liver lipid content

High fructose diets are known to cause increased lipid synthesis and storage by the liver, viscera and muscles (Le *et al.*, 2009; Bergheim *et al.*, 2008; Le *et al.*, 2006). In the current study administration of PC + FS and LD + FS for 12 weeks resulted in increased hepatic lipid content in growing Wistar rats (Figure 4.5B). This finding suggests that the 12% fructose solution induced increased lipid storage by the liver which can potentially cause fatty liver disease. Fatty liver disease is a liver-based manifestation of the development of metabolic syndrome (Song *et al.*, 2013; de Moura *et al.*, 2009). Fenofibrate is a lipid lowering drug (Staels *et al.*, 1998) and has been shown to reduce hepatic lipid synthesis and storage in 10-week old Wistar rats (Ferreira *et al.*, 2008). The current study reports similar results, that fenofibrate inhibited high fructose diet induced lipid accumulation in the liver (Figure 4.5B). *In vitro* studies have shown that triterpene saponin extracted from *T. sericea* root has lipolysis promoting properties (Mochizuki and Hasegawa 2006). In the current study the high dose of aqueous *T. sericea* leaf extract inhibited the fructose diet induced accumulation of lipids in the liver (Figure 4.5B) suggesting that at a high dose, the

aqueous *T. sericea* leaf extracts might prevent accumulation of lipids in the liver, thus exhibiting protective potential against diet induced fatty liver disease.

5.6.3 Markers of liver function

Alanine aminotransferase (ALT) is a surrogate biomarker for intra-hepatic damage (Thulin *et al.,* 2008). Alkaline phosphatase (ALP) is a biomarker that indicates post-hepatic liver damage or increased bone formation if plasma concentrations are elevated (Pratt and Kaplan, 2000; Reichling and Kaplan, 1988). Bilirubin is processed to a soluble state by the liver and then excreted in urine and faeces. Elevated concentrations of bilirubin can be indicative of red blood cell hemolysis or liver damage (Pratt and Kaplan, 2000). Albumin and globulin are major serum proteins that are synthesized by the liver. Decreased albumin and globulin serum concentrations are indicative of end-stage liver disease amongst other diseases (Oettl *et al.,* 2013).

In rats, high fructose diets have been reported to induce non-alcoholic fatty liver disease (NAFLD). The latter (NAFLD) has been implicated as one of the causes of increased serum ALT transaminase (de Catro *et al.,* 2013). Interestingly, while other research has noted that high fructose diets led to increased fat deposition in the liver and subsequent NAFLD (Castro *et al.,* 2011; Kanuri *et al.,* 2011), it could be inferred from this current study that although the feeding of a 12% fructose solution to growing Wistar rats for 12 weeks caused “fatty” livers, it did not result in increased serum ALT and ALP concentrations (Table 4.5).

The administration of fenofibrate has been shown to cause an increase in serum ALT and ALP concentration (Hajdu *et al.,* 2009; Ho *et al.,* 2004) due to hepatocellular damage (Tolman, 2000). Interestingly, Cindoruk *et al*. (2007) reported that administration of fenofibrate reduced serum total bilirubin, ALT and ALP in adult male Wistar rats with obstructive liver disease. Results of the current study showed that the administration of fenofibrate (100 $mg.kg^{-1}$ body mass per day) for 12 weeks to growing Wistar rats fed a 12% fructose solution caused increased serum ALP concentration (Table 4.5), thus suggesting possible hepatic damage by fenofibrate. However, that fact that other serum markers of liver function (ALT, total bilirubin, albumin and globulin) were not affected suggest that the elevated ALP could have been from bone or intestinal cells (Wolf, 1999), since high bone turnover in growing animals is associated with increased serum concentrations of ALP (Pratt and Kaplan, 2000; Wolf, 1999; Reichling and Kaplan, 1988). Syversen *et al.* (2009) reported that PPARα increases osteoblast activity which in turn results in elevated blood ALP. An isoenzyme of ALP is also found in intestinal cells (Wolf, 1999), thus the increased blood ALP in rats given FF + FS could have been due to the increased intestinal tissue which was noted in the current study. The specific isoenzyme of ALP that was increased was not identified in the current study. Further investigations are required to identify the possible source (liver, bone and liver) of the increased blood ALP concentration following fenofibrate administration to growing rats.

Terminalia species (*T. chebula*, *T. arjuna* and *T. brownii*) have hepatoprotective properties and are used in ethnomedicine to treat liver diseases (Basha *et al.,* 2012; Manna *et al.,* 2006; Tasduq *et al.,* 2006). Manna *et al.* (2006) showed that aqueous extracts of *T. arjuna* stem bark lowered serum ALP in Swiss-albino rats with carbon tetrachloride induced liver injury. In the current study the low and high dose of aqueous *T. sericea* leaf extracts had no

effect on serum albumin, ALT and ALP concentrations of growing Wistar rats fed 12% fructose solution, which suggests that the oral administration of aqueous *T. sericea* leaf extracts did not cause liver damage further confirming the *in vitro* findings using the brine shrimp nauplii.

5.6.4 Markers of kidney function

Creatinine and blood urea nitrogen (BUN) are excreted by the kidney (Chen *et al.,* 2011b). Elevated serum concentrations of creatinine and BUN indicate renal complications or increased muscle catabolism (Chen *et al.,* 2011b; Cavalcanti *et al.,* 2006). In adult Sprague-Dawley rats high fructose (60% w/w) feeds consumed over a 6-week period have been demonstrated to cause kidney damage and decrease their ability to filter, thus increasing blood creatinine and BUN concentrations (Gersch *et al.,* 2007). In the current study the administration if 12% fructose solution for 12 weeks had no effect on serum creatinine and BUN, suggesting that the renal function of the rats' kidneys (across treatments) was not compromised.

Chronic use of fenofibrate is known to increase serum creatinine and BUN in humans (Chen *et al.,* 2011b). However the mechanisms by which it causes this are still not fully understood. Researchers have suggested that fenofibrate possibly reduces glomerular filtration (Chen *et al.,* 2011b). In the current study there was no elevation of serum creatinine and BUN concentrations from the growing Wistar rats following administration of fenofibrate (100 $mg.kg^{-1}$ body mass per day) for 12 weeks, suggesting that fenofibrate did not reduce glomerular filtration.

Terminalia arjuna aqueous stem bark extract has been shown to protect the kidneys against oxidative stress induced damage (Manna *et al.,* 2006) whilst *Terminalia bellerica* organic fruit extract administration to adult Streptozotocin diabetic rat for 60 days, reduced serum creatinine and urea (Latha and Daisy, 2010). In the current study, administration of aqueous *T. sericea* leaf extracts for 12 weeks did not affect serum creatinine and BUN in growing Wistar rats suggesting that *T. sericea* aqueous leaf extracts had no adverse effects on kidney function.

5.6.5 Effect on serum calcium and phosphorus concentrations

Calcium and phosphorus can be absorbed from the GIT or kidneys and are involved in bone formation and mineralization**.** Their serum concentration are mainly regulated via parathyroid and calcitonin hormone but are also be influenced by diet (de Paula and Rosen, 2010). Calcium and phosphorus concentrations can thus be indicative of bone activity (resorption and formation), GIT mineral absorption and kidney mineral absorption and secretion. Fructose has been shown to cause kidney damage that can result in increased calcium and phosphorus excretion (Taylor and Curhan, 2008; Levin *et al.,* 2007). In the current study the administration of PC + FS did not result in increased excretion of the two minerals suggesting that there was no damage to the nephrons. However results of the current study show that the administration of fenofibrate (100 $mg.kg^{-1}$ body mass per day) for 12 weeks to growing Wistar rats fed a 12% fructose solution resulted in increased serum phosphorus concentration (Table 4.5). This could have been due to increased absorption in GIT and or reabsorption in the kidney. However, further research needs to be

conducted to investigate mechanisms by which fenofibrate increased serum phosphorus. Tannins are known to have potential to reduce the absorption of minerals (calcium and phosphorus) and proteins from the GIT (Muhammad and Oloyede, 2004). Although in the current study it was qualitatively established that the aqueous *T. sericea* leaf extracts contained tannins, the similarity in the serum calcium and phosphorus concentration in rats which were given both a low and high dose of the aqueous *T. sericea* leaf extracts for 12 weeks suggests that the tannins in the extract did not negatively affect their absorption from the GIT.

5.6.6 Effect on serum cholesterol

Elevated serum cholesterol levels are associated with various systemic diseases such as metabolic syndrome, diabetes mellitus and cardiovascular diseases (Neuschwander-Tetri and Wang, 2013). While the oral administration of a 21% fructose solution in 8-week old Wistar rats for 8 weeks caused elevated serum cholesterol concentrations (Yadav *et al.,* 2007), in the current study the oral administration of 12% fructose solution for 12 weeks did not cause hypercholesterolaemia in growing Wistar rats. Fenofibrate is known to reduce serum cholesterol by inhibiting lipid absorption in the gut and promoting lipid oxidation (Valasek *et al.,* 2007; Jeong *et al.,* 2004b). In the current study, no changes in serum cholesterol concentration were observed following the oral fenofibrate administration for 12 weeks in growing Wistar rats given a 12% fructose solution. Saponins and tannins from *Terminalia arjuna* are known to lower cholesterol synthesis by inhibiting lipid absorption and synthesis in the gut and liver, and by increasing high density lipoprotein synthesis (Ram *et al.,* 1997; Tiwari *et al.,* 1990). *T. sericea* aqueous leaf

extracts that were administered to growing Wistar rats for 12 weeks contained saponins and tannins; however they did not affect serum cholesterol concentrations. It should be noted that the effects of the treatments on serum cholesterol could have been affected by the fact that the rats were not on a high fat diet.

5.6.7 Effect on serum amylase activity

Amylases which catalyse the hydrolysis of starch and glycogen to glucose are found in saliva and exocrine pancreatic secretions. Their serum concentrations can be used as markers of inflammation of the salivary gland or pancreas (Huang *et al.*, 2004; Pieper-Bigelow *et al.*, 1990). Amylases are targets for diabetes medications: inhibition of amylases reduces glucose absorption and thus reducing hyperglycaemia (Hasenah *et al.*, 2006). Huang *et al.* (2004) observed that in 7-week old Sprague-Dawley rats given a high-fructose (60%) diet for 8 weeks did not affect serum amylase concentrations. Similarly results of the current study show that oral administration of 12% fructose solution for 12 weeks had no effect on serum amylase concentration in growing Wistar rats. This suggests that 12% fructose solution did not cause inflammation to the salivary glands and pancreas. Furthermore administration of fenofibrate had no effect on the amylase concentration in growing Wistar rats, suggesting that oral administration of fenofibrate did not cause pathology in salivary glands and or that of the pancreas. While findings from the *in vitro* research done by Nkobole *et al.* (2011) showed that *T. sericea* stem bark tannins, saponins and flavonoids inhibit amylase, in the current study, *in vivo*, aqueous *T. sericea* leaf extracts had no effect on amylase serum concentration in growing Wistar rats. The effects of *T. sericea* on amylases and carbohydrate absorption *in vivo* need to be further investigated.

CHAPTER 6: CONCLUSION AND RECOMENDATIONS

6.0 Conclusion and recommendations

The study evaluated the phytochemical composition of aqueous *T. sericea* leaf extracts, toxicity of the aqueous *T. sericea* leaf extract *in vitro*, effects of the administration of the aqueous leaf extracts on the growth performance, glucose tolerance and HOMA-IR index, GIT viscera and other visceral organs, hepatic metabolite storage and the general health profile of growing male Wistar rats fed 12% fructose solution. Using qualitative methods, *T. sericea* leaf extracts were found to contain pharmacologically active phytochemicals and to be non-toxic to Brine shrimp nauplii. Future studies should quantify the phytochemical constituents of the aqueous *T. sericea* leaf extracts and investigate the toxicity of aqueous *T. sericea* leaf extracts *in vivo*.

Findings of the current study showed that while the oral administration of a 12% fructose solution for 12 weeks to growing male Wistar rats resulted in the development of a "fatty liver", one of the markers of metabolic syndrome, it did not cause the development of hyperglycaemia, hypertriglyceridaemia, hypercholesterolaemia, insulin resistance and central obesity, which are the other markers of metabolic syndrome. The administration fenofibrate and high dose of *T. sericea* extracts protected against the development of a high fructose diet-induced fatty liver. The mechanisms of how the high dose of *T. sericea* extracts protected against development of a "fatty liver" need to be further investigated in non-alcoholic fatty liver disease and obesity models. The protective effects of aqueous *T. sericea* leaf extracts can be further exploited clinically in the prevention and possibly treatment of patients with fatty liver disease. The oral administration of a low dose (100 $mg.kg^{-1}$ body mass per day) and high dose (400 $mg.kg^{-1}$ body mass per day) of aqueous *T.*

sericea leaf extracts did not negatively affect growth performance (body mass, empty carcass mass and indexes of long bone growth).

While the administration of aqueous *T. sericea* leaf extracts did not improve glucose handling following an oral glucose challenge, the extracts had no adverse effect on the rats' glucose tolerance, plasma insulin concentration, visceral organ morphometry and on markers of general health profile of the rats. These observations suggest that aqueous *T. sericea* leaf extracts could be used in ethnomedicine without an increased risk of compromising the glycaemic control, growth performance, GIT and other viscera's morphometry and general health. The oral administration of fenofibrate (100 $mg.kg^{-1}$ body mass per day) to growing Wistar rats fed a 12% fructose solution for 12 weeks reduced empty carcass, reduced linear growth of the tibia, caused hyperglycaemia, possible liver injury and increased mass of visceral organs (small intestine, liver and kidney) in growing Wistar rats. The adverse effects of the chronic administration of fenofibrate on tibia bone growth, blood glucose homeostasis and liver function enzymes need to be further investigated at a cellular and molecular level.

6.1 Limitations

In the current study administration of 12% fructose solution *ad libitum* resulted in increased liver lipid ("fatty liver") however, no other signs of metabolic dysfunction in growing Wistar rats were observed possibly due to it not providing enough excess calories. Therefore future studies should consider using fructose solution at a higher concentration and possibly supplementing the standard rat chow with lard, to increase calorie and fat intake. The present study used male rats only, future studies should consider investigating

the effects of orally administered aqueous *T. sericea* extracts and fenofibrate in male and female rats fed normal rat chow and plain water as this will allow for an investigation of the effects of the extracts on normal rats of different rat genders. They (future studies) should also consider investigating and comparing the effects of *T. sericea* organic extracts to aqueous extracts. Additionally, future studies should also investigate the effects of oral administration of aqueous *T. sericea* leaf extracts on nutrient absorption from the GIT. While the current study did not employ molecular techniques to elucidate some of the mechanisms that may have been at play, it is recommended that future studies include molecular techniques in order to get measurements gene and protein expression level as this would helps better explain the mechanisms behind the findings of the study. Other technical limitations include that only qualitative and not quantitative phytochemical analyses were performed on *T. sericea* leaf extracts and that subjective assessment of radiographs was done to analyse bone density of the rats. Future studies should perform quantitative analyses on *T. sericea* leaf extracts and also do densitometry to analyse bone density of the rats. While it would have been interesting to investigate the effects of aqueous *T. sericea* leaf extracts alone, the current study was more focused on the effects of fructose induced changes as opposed to general effects of aqueous *T. sericea* leaf extracts which would have required a different experimental design.

CHAPTER 7: REFERENCES

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APPENDICES

Appendix 1

AESC3

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/29/05

APPLICANT: **Mr B W Lembede**

DEPARTMENT: **Physiology**

PROJECT TITLE: **Effect of dietary *Terminalia sericea* leaf aqueous extracts on the development of high-fructose diet-induced metabolic dysfunction in growing Wistar rats.**

Number and Species

40 Wistar Rats

Approval was given for the use of animals for the project described above at an AESC meeting held on **26 June 2012**. This approval remains valid until **30 June 2014**.

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 to the following additional conditions:

Conditions:

- A score sheet to be used to log the health of the rats.

Signed: _____ (Chairperson, AESC) Date: 31/7/12.

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

Signed: _____ (Registered Veterinarian) Date: 31/7/12

cc: Supervisor:
Director: CAS

6 REAGENTS 1 X 96 KIT

Each High Range Rat Insulin ELISA kit contains reagents for 96 wells, sufficient for 42 samples and one standard curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers.

The expiry date for the complete kit is stated on the outer label.

The recommended storage temperature is 2 °C - 8 °C.

Coated Plate	1 plate	96 wells	Ready for use
(mouse monoclonal anti-insulin)		8-well strips	

For unused microplate strips, reseal the bag using adhesive tape, store at 2–8°C and use within 8 weeks.

Calibrators 1, 2, 3, 4, 5	5 vials	1000 µL	Ready for use

Rat Insulin

Color coded yellow

Concentration stated on vial label.

Calibrator 0	1 vial	5 mL	Ready for use

Color coded yellow

Enzyme Conjugate 11X	1 vial	600 µL	Preparation, see below

Peroxidase conjugated mouse monoclonal anti-insulin

Enzyme Conjugate Buffer	1 vial	6 mL	Ready for use

Color coded blue

Wash Buffer 21X	1 bottle	50 mL	Dilute with 1000 mL redistilled water to make wash buffer 1x solution

Storage after dilution: 2 °C - 8 °C for 8 weeks

Substrate TMB	1 vial	22 mL	Ready for use

Colorless solution *Note! Light sensitive!*

Stop Solution	1 vial	7 mL	Ready for use

0.5 M H_2SO_4

6.1 Preparation of enzyme conjugate 1X solution

Prepare the needed volume of enzyme conjugate 1X solution by dilution of Enzyme Conjugate 11X (1+10) in Enzyme Conjugate Buffer according to the table below.

When preparing enzyme conjugate 1X solution for the whole plate, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11x vial. Mix gently.

Number of strips	Enzyme Conjugate 11X	Enzyme Conjugate buffer
12 strips (1 plate)	1 vial	6 mL
6 strips	300 µL	3 mL
4 strips	200 µL	2 mL

Storage after dilution: 2 °C - 8 °C for 8 weeks.

7 SPECIMEN COLLECTION AND HANDLING

Serum

Collect blood by venipuncture, allow to clot, and separate the serum by centrifugation.

Samples can be stored at 2 °C - 8 °C up to 24 hours.

For longer periods store samples at -20 °C. Avoid repeated freezing and thawing.

Plasma

Collect blood by venipuncture into tubes containing heparin or EDTA as anticoagulant, and separate the plasma fraction.

Samples can be stored at 2 °C - 8 °C up to 24 hours.

For longer periods store samples at -20 °C. Avoid repeated freezing and thawing.

7.1 Preparation of samples

No dilution is normally required, however, samples containing >150 µg/L should be diluted 1/10 v/v with Calibrator 0.

Note! Buffer containing sodium azid (NaN_3) can not de used for sample dilution.

8 TEST PROCEDURE

Prepare a calibrator curve for each assay run. All reagents and samples must be brought to room temperature before use.

1. Prepare enzyme conjugate 1X solution (according to the tables on previous pages), wash buffer 1X solution and samples.
2. Prepare sufficient microplate wells to accommodate Calibrators and samples in duplicate.
3. Pipette 10 µL each of Calibrators and samples into appropriate wells.
4. Add 50 µL of enzyme conjugate 1X solution into each well.
5. Incubate on a plate shaker (700-900 rpm) for 2 hours at room temperature (18 °C – 25 °C).
6. Wash 6 times with 700 µL wash buffer 1X solution per well using an automatic plate washer with overflow-wash function. Do not include soak step in washing procedure.
   Or manually,
   Discard the reaction volume by inverting the microplate over a sink. Add 350µL wash buffer 1X solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing procedure.
7. Add 200 µL Substrate TMB into each well.
8. Incubate 15 minutes at room temperature (18 °C – 25 °C).
9. Add 50 µL Stop Solution to each well.
   Place the plate on the shaker for approximately 5 seconds to ensure mixing.
10. Read optical density at 450 nm and calculate results.
    Read within 30 minutes.

Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.

9 INTERNAL QUALITY CONTROL

Commercial controls and/or internal serum pools with low, intermediate and high insulin concentrations should routinely be assayed as unknowns, and results charted from day to day. It is good laboratory practice to record the following data for each assay: kit lot number; dilution and/or reconstitution dates of kit components, OD values for the blank, Calibrators and controls..