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# Effect of zingerone on high fructose diet-induced metabolic derangements in growing Sprague-Dawley rats

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# EFFECT OF ZINGERONE ON HIGH-FRUCTOSE DIET-INDUCED METABOLIC DERANGEMENTS IN GROWING SPRAGUE-DAWLEY RATS

**Nondumiso Immaculate Lushozi**

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

Johannesburg 2020

**Declaration**

I, **Nondumiso Immaculate Lushozi**, 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. I certify that all the experimental procedures used in this dissertation were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2017/010/71B).

**_____**

**Nondumiso Lushozi**

**_____day of _____20_____at_____**

**Dedication**

To the loving memory of my late mother and great-grand mother

-Dudu Princess Kubheka

1979-2016

-Mildred Langa

1933-2016

**Research output**

CONFERERENCE PRESENTATION

**Lushozi N.I.,** Lembede, W and Chivandi, E. 2018. Effect of zingerone of zingerone on high-fructose diet-induced metabolic derangements in growing Sprague-dawley rats. Oral presentation at the University of the Witwatersrand, Post graduate expo, 6 September 2018, Johannesburg, South Africa.

**Abstract**

The consumption of fructose increases the risk of obesity, non-alcoholic fatty liver disease(s) (NAFLD) and metabolic syndrome in children. Zingerone, a phytochemical largely found in ginger, has antidiabetic and anti-obesogenic properties. This study investigated the potential of orally administered zingerone to protect growing Sprague-Dawley rats against dietary fructose induced metabolic derangements. Seventy-seven (38 male; 39 female) Sprague-Dawley rats in an interventional study, were randomly allocated to four treatment regimens which were administered for 12 weeks. The administered treatment regimens were as follows: regimen I: standard rat chow (SR) + plain water (PW) + plain gelatine cube (PC), regimen II: SR + 20% (w/v) fructose solution (FS) + PC, regimen III: SR + FS + 100 mg/kg/day of fenofibrate in gelatine cube and regimen IV: SR+ FS + 20 mg/kg/day of zingerone in gelatine cube. Two days before euthanasia the rats' tolerance to an oral glucose load was determined. At study termination following a 12-hour fast, but with access to drinking water, the rats' body weights were determined. Blood was collected, blood glucose concentration measured and plasma harvested. Following dissection the empty carcass weight, tibiae length, weight, and weight to length ratio were determined. Liver and visceral fat weights were measured. Plasma AST activity as well as triglyceride, cholesterol, insulin and adiponectin concentrations were determined. HOMA-IR was calculated. The liver lipid content and histology were determined. Fructose consumption significantly increased ($P < 0.05$) hepatic lipid accretion and caused hepatic steatosis but did not ($P>0.05$) have an effect on tolerance to an oral glucose challenge, body weight, empty carcass weight, tibiae indices, circulating metabolite (glucose, triglyceride, insulin and adiponectin) concentrations. Zingerone did not prevent the increased liver lipid accretion in the high-fructose diet-fed rats, but it prevented the development of steatosis in male and female rats. Fenofibrate mitigated the fructose-induced increase in liver lipid accretion and prevented steatosis but it caused glucose and insulin-related disturbances in male rats and increased ($P < 0.05$) plasma triglycerides in female rats. Fenofibrate also significantly increased ($P < 0.0001$) liver weights in male and female rats. Zingerone may potentially be used in the prevention of fructose diet-induced hepatic steatosis but the mechanism(s) of it potential beneficial effects require further interrogation.

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**List of abbreviations**

| | |
|---|---|
| ATP | Adult Treatment Panel III |
| ALT | Alanine aminotransferase |
| ALT | Analysis of variance |
| AST | Aspartate aminotransferase |
| AUC | Area under the curve |
| BMI | Body mass index |
| BW | Body weight |
| ChREBP | Carbohydrate response element-binding protein |
| CAS | Central Animal Services |
| COD-PAP | Cholesterol oxidase p-aminophenol |
| DALY | Disability-adjusted life-years |
| DHAP | Dihydroxyacetone phosphate |
| AMPK | Adenosine monophosphate -activated protein kinase |
| ELISA | Enzyme-linked immunosorbent assays |
| GGT | Gamma-glutamyl transferase |
| GDM | Gestational diabetes mellitus |
| GMO | Genetically modified foods |
| GLUT2 | Glucose transporter 2 |
| G3P | Glyceraldehyde-3-phosphate |
| GPO-PAP | Glycerol-3-phosphate oxidase-phenol + aminophenazone |
| HNF-4 | Hepatocyte Nuclear Factor 4 |
| HOMA-IR | Homoeostasis model assessment of insulin resistance |
| IDF | International Diabetes Federation |
| IL-6 | Interleukin-6 |
| IDF | International Diabetes Foundation |
| IRS-1 | Insulin receptor substrate 1 |
| DHAP | Isomers dihydroxyacetone phosphate |
| MetS | Metabolic syndrome |
| NCEP | National Cholesterol Education Program |
| NHANES | National Health and Nutrition Examination Survey |
| NAFLD | Non-alcoholic fatty liver disease |
| NAS | Non-alcoholic fatty liver disease activity score |
| NASH | Non-alcoholic steatohepatitis |
| NF-$\kappa$B | Nuclear factor kappa-light-chain-enhancer of activated B cells |

| | |
|---|---|
| PPAR-α | Peroxisome proliferator-activated receptor-α |
| ROS | Reactive oxygen species |
| SCD-1 | Stearoyl-CoA desaturase-1 |
| SD | Standard deviation |
| SREBP-1 | Sterol regulatory element-binding transcription factor 1 |
| STZ | Streptozotocin |
| TGFβ1 | Transforming growth factor beta1 |
| TLr | Relative to tibia length |
| TNBS | Trinitrobenzene sulphonic acid |
| TNFα | Tumour necrosis factorα |
| T-I-DM | Type-I-diabetes mellitus |
| T-II-DM | Type II diabetes mellitus |
| VLDL | Very low-density lipoprotein |
| WHO | World Health Organization |

# Chapter One: Introduction and Justification

## 1.1 Introduction

The world is in the midst of global epidemics inclusive of these epidemics are obesity, type II diabetes (T-II-DM), and metabolic syndrome (Ussar et al., 2015). Obesity refers to abnormal or excessive body fat accretion to the degree that it may cause unfavourable effects on the well-being of the individual (Hermanus et al., 2015). It is estimated that 42 million children globally under the age of five are overweight or obese (Walker et al., 2018). In South Africa, the prevalence of childhood obesity is approximated to be 7.8% and it is predicted to escalate to 22.8% by 2020 (Toriola et al., 2012). The increase in childhood obesity has been identified as the main driver for the increase in the prevalence of metabolic syndrome (MetS). Metabolic syndrome is the cluster manifestation of metabolic derangements inclusive of visceral obesity, hyperglycaemia, insulin resistance and dyslipidaemia (Mameli et al., 2017).

The latter (MetS) increases the risk of developing type II diabetes mellitus (T-II-DM) and cardiovascular diseases which are amongst the forefront causes of death worldwide (Leon and Maddox, 2015). According to International Diabetes Federation (IDF) the global prevalence of diabetes mellitus was 8.8% as of 2015 and is expected to increase to 10.4% by 2040 (Ogurtsova et al., 2017). Additionally, it is estimated that globally, one billion people are have MetS (Saklayen, 2018). Recent studies have suggested that non-alcoholic fatty liver disease (NAFLD) should be considered the hepatic manifestation of MetS (Alisi et al., 2012; Kim et al., 2014). Obesity and insulin resistance which are key indicators of MetS are also seen in the accumulation of fat in the liver (Lallukka et al., 2013) showing the link between MetS and NAFLD. However, Han et al. (2015) demonstrated that NAFLD can occur independently of MetS.

Non-alcoholic fatty liver disease is a spectrum of liver disease characterised by increased infiltration and accumulation (>5% wet liver weight) of lipids inside the liver parenchymal cells, in the absence of excessive alcohol intake (Petäjä and Yki-Järvinen, 2016). The pathological manifestations of NAFLD range from simple steatosis (NAFLD), non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis (Jiang et al., 2014), which in some cases may progress to hepatocellular carcinoma or liver failure (Athyros et al., 2015). Simple steatosis (NAFLD) is reversible, however, if left untreated it will progress to NASH that is characterised by hepatocellular necroinflammation and ballooning (Athyros et al., 2017). Similar to NAFLD, NASH is also reversible but will progress to cirrhosis if left untreated (Mattar et al., 2005; Brown and Kleiner, 2016).

It is estimated that approximately one billion of the global population has NAFLD (Loomba and Sanyal, 2013). However, NAFLD is progressive in 20% of the general global population (Nair et al., 2004; Loomba and Sanyal, 2013). Obesity is positively correlated with NAFLD. Approximately, 70% and 80% of obese children and adults have NAFLD, respectively (El-Koofy et al., 2012; Gaggini et al., 2013) The growing prevalence of obesity, MetS and NAFLD has been attributed to increased adoption of sedentary lifestyles and the consumption of diets that are rich in fructose (Sahoo et al., 2015; Lozano et al., 2016). The 1999–2004 data from the National Health and Nutrition Examination Survey (NHANES) indicated that the average daily intake of fructose in the United States is now approximately 49 g per capita, which is equivalent to 9.1% of total daily caloric intake (Klein and Kiat, 2015).

Fructose is the sweetest monosaccharide which can be found in honey, vegetables and fruits (Das, 2015; Sluik et al., 2015). The metabolism of fructose that occurs mainly in the liver bypasses the rate-limiting steps of glycolysis and thus results increased *de novo* lipogenesis (Bidwell et al., 2017; de Castro et al., 2013; Hannou et al., 2018). The increased *de novo* lipogenesis leads to increased lipid accumulation in the liver parenchymal cells and in peripheral tissues and subsequently the manifestation of obesity, NAFLD and MetS (Alwahsh and Gebhardt, 2017; Friedman et al., 2018; Hannou et al., 2018).

A multitude of synthetic pharmacological agents can be used to manage elements of obesity, NAFLD and MetS (Lonardo et al., 2017; Kushner, 2018). However, synthetic pharmaceutical agents generally are mono-therapeutic, expensive, inaccessible to the global population and are associated with adverse effects (Strang et al., 2012). Phentermine, an anti-obesogenic drug, for example, has been shown to increase heart rate, blood pressure, and to cause dryness of the oral cavity, insomnia, constipation and to worsen anxiety and irritability (Srivastava et al., 2019). Thus, as a consequence, approximately 80% of the global population prefers to use plant-derived ethnomedicines to treat and or manage multifaceted systemic ailments such as obesity, MetS and NAFLD (Alves and Rosa, 2007). Ethnomedicines such as ginger (*Zingiber officinale*) rhizome, a commonly consumed spice worldwide, contain bioactive phytochemicals such as gingerol, shagoals and vanillylacetone which is also known as zingerone (Ogunyinka et al., 2015).

Zingerone is a nontoxic (Rehman et al., 2019) semi-water soluble phytochemical that possesses anti-inflammatory, antioxidant, antithrombotic, antidiabetic and anti-obesogenic properties (Srinivasan, 2005). The mechanism by which zingerone exerts its antidiabetic and anti-

obesogenic effects is through the upregulation of norepinephrine-mediated lipolysis. *In vivo*, the oral administration of zingerone at 170 mg/kg was reported to reduce fasting blood glucose and adiposity in ovariectomized rats (Han et al., 2008). *In vitro* studies have shown that zingerone improves the vascular activity in isolated arteries from streptozotocin-diabetic male rats (Ghareib et al., 2016). A study by Banji et al. (2014) revealed that the antioxidant boosting activities of zingerone can be used to prevent oxidant-stress-related disorders such as MetS, NAFLD and T-II-DM.

### 1.2 Justification

Most studies investigating the pathophysiology of diet-induced obesity, MetS and NAFLD use adult rat and mouse models (de Sousa Rodrigues et al., 2017; Han et al., 2008). Nonetheless, the problem of obesity, MetS and NAFLD is growing in children as a consequence of poor dietary habits (Pacifico et al., 2011; Güngör, 2014). Most studies investigating metabolic disorders generally have a bias for using male rat and mouse models (Giles et al., 2016). Empirical evidence suggests that males and females respond differently to dietary and medicinal interventions (Ren and Kelley, 2009; Williams et al., 2015; Arno and Thomas, 2016). Thus, there is a need for investigating both genders under the same conditions of dietary and medicinal interventions.

Furthermore, several studies which investigated the effect of zingerone against obesity, MetS and NAFLD focused on using chemically-induced diabetic adult rats models and ovariectomized models of obesity (Ghareib et al., 2016; Han et al., 2008). However, there is no research that has been done to evaluate the potential protective effects of zingerone against diet-induced metabolic disorders in children. Therefore, this study interrogated the effect of orally administered zingerone on metabolic derangement parameters of high-fructose diet-fed growing male and female rats, modelling children fed obesogenic diets.

### 1.3 Aim and objectives

The aim of the current study was to investigate the potential of orally administered zingerone to protect growing Sprague-Dawley rats against high-fructose diet-induced metabolic derangements. Specifically, the study interrogated the effect of zingerone on:

a) growth performance as determined by body weight andlong bone indices and the ability to tolerate an oral glucose load.

b) circulating metabolic substrate (blood glucose, cholesterol and triglycerides) concentration.

c) the concentration of insulin and adiponectin; hormones that regulate metabolism.

d) insulin sensitivity as determined by the homoeostasis model assessment of insulin resistance.

e) liver mass and lipid content, liver histology, non-alcoholic fatty liver disease activity score and surrogate markers (plasma aspartate aminotransferase activity) of liver function.

### 1.4 Hypothesis

$H_0$: Orally administered zingerone does not protect growing male and female Sprague-Dawley rats against high-fructose diet-induced metabolic derangements.

$H_1$: Orally administered zingerone protects growing male and female Sprague-Dawley rats against high-fructose diet-induced metabolic derangements.

# Chapter Two: Literature Review

## 2.1 Obesity

Obesity refers to the abnormal or excessive accumulation of body fat that imposes a risk to the health status of an individual (Grundy, 2004). The literature further elucidates that obesity can be subdivided according to fat distribution in the body (Abu-Abid et al., 2002; Aucouturier et al., 2009). Central (android) obesity which is typified by immoderate accrual of fats in the abdominal cavity and is positively correlated with metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD), type-II-diabetes mellitus (T-II-DM) and cardiovascular complications (McTernan et al., 2002; Tankó and Christiansen, 2006). In adults general obesity is assessed by measuring body mass index [BMI; weight (kg)/height ($m^2$)] (Flegal et al., 2010). In adults a BMI greater than 25 $kg/m^2$ denotes overweight (Flegal et al., 2013). However, the limitations of using BMI to assess obesity is that it does not take into account muscle mass, bone density, adipose tissue distribution and overall body composition (Haby et al., 2006; Gurunathan and Myles, 2016). Central obesity is assessed using waist circumference as well as waist to hip circumference in adults (Ahmad et al., 2016). The cut-off points for waist circumference are 94 cm and 80 cm in male and females, respectively (Ahmad et al., 2016). On the other hand, waist to hip ratios that are >1 and > 0.85 indicate visceral obesity in males and females, respectively (Ahmad et al., 2016). In children, obesity is assessed using growth charts and z-scores (Khadilkar et al., 2015). When using growth charts to assess obesity, a bodyweight within the 85th to 94th percentile for age and sex indicates overweight and a bodyweight above the 95% for age and sex indicates obesity (Freedman et al., 2009). The world health organisation (WHO) defines a z-score >2 indicates overweight and a z-score >3 indicates obesity (Wang and Chen, 2012).

Childhood obesity has emerged as a pertinent global health concern (Karnik and Kanekar, 2015; Ng et al., 2014). It is estimated that one out of three children in the United States is overweight or obese (Kumar and Kelly, 2017). Data from empirical research suggests that the global prevalence of childhood obesity has increased by 47.1% in the past three decades (Ng et al., 2014; Ogden et al., 2016) and it is projected that by 2025, 91 million children aged between 5-17 years will be obese (Lobstein and Jackson-Leach, 2016). Furthermore, the WHO reported that in 2016, ~1.9 billion adults (≥18 years and older) were overweight or obese (World Health Organization, 2018)(World Health Organization, 2018). The adoption of unhealthy dietary habits coupled with sedentary lifestyles have been identified as the key propellants of the obesity epidemic (World Health Organization, 2018). Other risk factors for childhood obesity include genetics, lack of sleep, gastrointestinal tract health (mucosal barrier

and gut microbiota), low self-esteem as well as biological and environmental stress (Ogden et al., 2016). In 2010, obesity was estimated to have caused 3.4 million deaths, and 3.8% of disability-adjusted life-years (DALYs) globally, (Ng et al., 2014). Studies have revealed that childhood obesity leads to the manifestation of various adverse health outcomes, including MetS, NAFLD and T-II-DM, in the subsequent developmental phases of life (Simmonds et al., 2016).

### 2.2 Metabolic syndrome

Worldwide, it is estimated that 20-25% of adults have MetS (Lone et al., 2017). Metabolic syndrome is defined as a cluster manifestation of cardio-metabolic risk factors, inclusive of abdominal obesity, insulin resistance, hyperglycaemia, dyslipidaemia (hypertriglyceridemia, hypercholesterolemia, hyper-low-density-lipoproteincholesterolemia and hypo-high-density-lipoprotein-cholesterolemia) and hypertension (Furukawa et al., 2004). Collectively, these cardio-metabolic risk factors excercerbate the odds of developing T-II-DM and cardiovascular disease (O'Neill and O'Driscoll, 2015). Obesity has been identified as the key mediator for the development of MetS, NAFLD, T-II-DM and cardiovascular disease (Ito et al., 2016; Ottobelli Chielle et al., 2016). According to the WHO, International Diabetes Foundation (IDF) and the American Association of Clinical Endocrinologists, MetS is diagnosed when a patient has central obesity, diabetes and any other three risk factors (Isomaa, 2003; Han and Lean, 2016). On the other hand, the National Cholesterol Education Program (NCEP) and Adult Treatment Panel III (ATP III) guidelines state that MetS is diagnosed when a patient has any three MetS risk factors (Han and Lean, 2016; Jama, 2001). Numerous studies have shown that MetS is synonymous with the onset of NAFLD (Marchesini et al., 2003; Rector et al., 2008). It is estimated that 65% of patients with MetS have NAFLD (Volynets et al., 2012).

### 2.3 Non-alcoholic fatty liver disease

The global prevalence of NAFLD is estimated to be 25-30% (Rowell and Anstee, 2015; Fattahi et al., 2016; Younossi et al., 2016, 2018). The highest prevalence of NAFLD has been reported in South America at 31% and in the Middle East at 32% (Younossi et al., 2018). Africa is reported to have the lowest prevalence of NAFLD at 14% (Younossi et al., 2016). The prevalence of childhood NAFLD is estimated to be 3–10% globally (Nobili et al., 2019).

Interestingly, NAFLD, which is the leading cause of liver failure in children (Loomba et al., 2009), is more prevalent in male children than in female children (Schwimmer et al., 2005).

Non-alcoholic fatty liver disease is a broad range of liver conditions including simple-fatty liver disease (NAFLD or simple steatosis), non-alcoholic steatohepatitis (NASH) and cirrhosis. These liver conditions manifest without the excessive consumption of alcohol or the use of steatotic medications (Kanuri and Bergheim, 2013). Simple-fatty liver (NAFLD) disease is characterised by hepatic fat infiltration and accumulation (Rowell and Anstee, 2015). It can be reversed through lifestyle adjustments (increased physical activity and the adoption of a healthy diet) and anti-steatotic medications (Jegatheesan and De Bandt, 2017). However, if NAFLD is untreated it will advance to NASH which is also reversible. Steatohepatitis is characterised by steatosis, liver inflammation and hepatocyte injury (Bedossa, 2017; Wong et al., 2016). Similarly, if NASH is not treated the inflammation will trigger the production of fibrotic tissue and consequently progress to cirrhosis that can culminate in hepatic carcinoma and or liver failure (Murillo et al., 2016).

The severity and progression of NAFLD can be assessed using the non-alcoholic fatty liver disease activity score (NAS), which is a semi-quantitative technique (Brunt et al., 2011). The NAS scoring criteria includes the grading of hepatic steatosis, lobular inflammation and hepatocyte ballooning (Angulo et al., 2015; Bedossa, 2016). A NAS score greater than five indicates NASH (Bedossa, 2017). Non-alcoholic fatty liver disease can also be assessed by evaluating the plasma activity and or concentration of liver function biomarkers (Anderson et al., 2015). The surrogate liver function biomarkers include aspartate aminotransferase, (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), bilirubin, albumin and ammonia (Anderson et al., 2015). However, simple fatty liver disease merely results in hepatic fat infiltration and accumulation without any hepatocellular damage and thus it generally does not alter the surrogate biomarkers of liver function (Barreyro et al., 2015; Valenti et al., 2016).

The main risk factors for developing NAFLD include obesity, insulin resistance, unhealthy dietary habits, gastrointestinal tract microbiota health, genetic predisposition MetS and T-II-DM (Kanuri and Bergheim, 2013). Interestingly, it is estimated that approximately 80-85% of patients affected by T-II-DM have NAFLD (Anstee et al., 2013).

## 2.4 Diabetes Mellitus

Diabetes mellitus is a metabolic disease that is characterised by hyperglycaemia and it is one of the leading causes of mortality and morbidity worldwide (Ezzati, 2016). Between 2012 and 2013, diabetes mellitus resulted in 1.5–5.1 million deaths per year (Tao et al., 2015). Globally it is estimated that 8% of children and 26% of adolescents have diabetes mellitus (Tao et al., 2015). The three types of diabetes mellitus are type-I-diabetes mellitus (T-I-DM), T-II-DM and gestational diabetes (Iyer et al., 2015).

Type I diabetes mellitus occur due to β-cell destruction and it leads to insulin deficiency and hyperglycaemia (Katsarou et al., 2017). Type II diabetes occurs due to the tissues' reduced ability to respond effectively to the actions of insulin (Magkos et al., 2016). Gestational diabetes mellitus (GDM), occurs during pregnancy when compensated insulin synthesis and secretion by pancreatic β-cells are insufficient for the insulin resistance and increased hepatic glucose production that takes place during pregnancy (Abell et al., 2015).

## 2.5 Rat models for investigating metabolic disorders

Metabolic disorders are increasing at an alarming rate, especially in children (Sacheck, 2008). This has resulted to the development of several animal models that can be used to interrogate the aetiology and pathophysiology of metabolic disorders as well as discovery and development interventions for metabolic disorders. The animal models for studying metabolic disorders include genetically-induced, chemically-induced, diet-induced and metabolic programming models (Bellamkonda et al., 2018; Rehman et al., 2019).

### *2.5.1 Genetically-induced models*

Genetically induced models of metabolic disorders are mainly used to investigate the aetiology and pathophysiology of metabolic disorders that are induced by single-gene mutation (Good, 2005). Monogenic animal models of metabolic diseases are crucial in understanding metabolic disorders that have single-gene aetiology (Kleinert et al., 2018). The most commonly used monogenic animal models for interrogating metabolic disorders are the leptin-deficient ob/ob mouse, leptin receptor-deficient db/db mouse, MC4 receptor-deficient rats and the Zucker obese rat model (Lutz and Woods, 2012). One of the main criticisms of monogenetic animal models is that in humans metabolic disorders are not typically caused by single-gene mutations (Kleinert et al., 2018). Therefore, monogenetic animal models do not

provide full insights on the aetiology and pathophysiology of metabolic disorders in humans that are commonly caused by diet (Nilsson et al., 2012).

### *2.5.2 Chemically-induced models*

In chemically-induced animal models of metabolic disorders, streptozotocin (STZ) is injected intraperitoneally to induce pancreatic β-cell dysfunction (Eleazu et al., 2013). Streptozotocin is a naturally occurring diabetogenic compound which enters the pancreatic β-cell via the Glucose transporter2 (GLUT2) transporters (Eleazu et al., 2013). Once inside the pancreatic β-cells, STZ causes cell destruction, therefore, decreasing insulin production and secretion (Eleazu et al., 2013). Therefore, chemically-induced animal models are appropriate for T-I-DM studies (Dhuria et al., 2015). However, T-II-DM is the most prevalent type of diabetes and it commonly occurs as a result of poor dietary habits and obesity (Li et al., 2015).

### *2.5.3 Diet-induced models*

The consumption of unhealthy diets contributes significantly to the exponential growth in the proportion of metabolic disorder cases (Hosseini et al., 2016). Thus most studies investigating metabolic disorders tend to use dietary models which are induced by feeding obesogenic diets (Lagisz et al., 2015). Importantly, diet-induced models mimic the pathophysiology that occurs in humans and thus they are preferred by most researchers interrogating metabolic disorders (Wang and Liao, 2012). The commonly utilised diet-induced rat models for metabolic disorders include the high-fat diet, high-carbohydrate diet, western-style (high-fat and high-carbohydrate) diet and high-fructose diet models (Odermatt, 2011; Kuate et al., 2015; Zhuhua et al., 2015).

## 2.6 Fructose in the development of metabolic disorders

Fructose is a monosaccharide that requires GLUT5 transporters to facilitate its absorption from the jejunum lumen into the enterocyte (Robinson et al., 2000). It is metabolised mainly in the liver, where fructokinase, phosphorylates fructose into fructose-1-phosphate (Nomura and Yamanouchi, 2012). The fructose-1-phosphate is then cleaved by aldolase B to produce the isomers dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) (Arakaki et al., 2009).

As a consequence of fructose metabolism bypassing the rate-limiting step of glycolysis, DHAP and G3P are used to synthesis triglycerides and very low-density lipoproteins (VLDLs) through *de novo* lipogenesis (Jia et al., 2014). The triglycerides can infiltrate and accumulate in the liver, thus causing NAFLD (Gaggini et al., 2013). Moreover, the triglycerides can be enclosed into VLDLs and be transported via circulation to peripheral tissues such as the visceral adipose tissue (Vergès, 2015). Therefore, the consumption of fructose augments hepatic *de novo* lipogenesis (King, 2012) and subsequently the manifestation of NAFLD, dyslipidaemia and visceral obesity (Gaggini et al., 2013).

Additionally, fructose upregulates the expression of lipogenic transcription factors including carbohydrate response element-binding protein (ChREBP) and sterol regulatory element-binding transcription factor 1 (SREBP-1); which contribute to the manifestation of metabolic disorders (Radovits et al., 2009). Visceral obesity has been shown to suppress the expression and secretion of adiponectin, which is an anti-inflammatory adipokine (Ohashi et al., 2014). Adiponectin is a polypeptide that increases insulin sensitivity by stimulating fatty acid oxidation in tissues by activating 5' AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-alpha (PPARα) (Viscarra et al., 2011; Ohashi et al., 2014). Thus, the hypoadiponectinemia that occurs due to visceral obesity leads to insulin resistance and the accumulation of lipids in the tissues (Di Chiara et al., 2012; Emanuela et al., 2012).

Obesity increase the secretion of adipokines and an increase in adipokine secretion have been linked to the inhibition of insulin action in peripheral tissues like the liver and skeletal muscle, but also to the development of diabetes-associated complications like insulin resistance (Barbosa et al., 2019). This increase in adipokines theirfore triggers chronic low-grade inflammation (Choi and Jung., 2014). Additionally, visceral obesity results in mild inflammation that is characterised by increased expression and secretion of pro-inflammatory adipokines including leptin, resistin, interleukin-6 (IL-6), tumour necrosis factorα (TNFα) (Anggraini et al., 2018; Kuryszko et al., 2016). Leptin is an adipocyte-derived hormone, which regulates food intake, body fat accumulation and metabolism (Bell and Rahmouni, 2016). Excessive secretion of leptin will result in hyperleptinemia and leptin resistance (Aizawa-Abe et al., 2000) which, consequently lead to the manifestation of obesity and insulin resistance (Sáinz et al., 2015; Yang et al., 2015).

The pro-inflammatory cytokines IL-6 and TNFα inhibit the phosphorylation of the insulin receptor substrate 1 (IRS-1) signalling adapter protein for insulin (Fasshauer and Paschke,

2003; da Costa et al., 2016). Consequently, this leads to insulin resistance, hyperglycaemia and hyperinsulinemia (Mukherjee et al., 2013) which favour lipogenesis and can cause the onset of dyslipidaemia (Jung and Choi, 2014). Dyslipidaemia increases the risk of developing atherosclerosis and numerous other cardio-metabolic conditions (Gaggini et al., 2013; Lonardo et al., 2013).

### 2.7 Interventions for metabolic disorders

The escalating prevalence of metabolic disorders globally has heightened the need to develop strategic interventions to help prevent and manage metabolic syndrome (Samodien et al., 2019). The first line of combat against metabolic syndrome is lifestyle change (Bassi et al., 2014). Lifestyle change encompasses increased physical activity and the adoption of healthy diets that are low in carbohydrate, fats and calories (Iqbal et al., 2010; Tobias et al., 2015). However, lifestyle change interventions are associated with poor compliance (Parajuli et al., 2014).

Synthetic pharmaceutical drugs such as fenofibrate and metformin have been shown to be effective at treating and managing specific elements of metabolic disorders (Rosenson, 2008; Mazza et al., 2012). While fenofibrate has been demonstrated to lessen hepatic lipid content and peroxidation (Harano et al., 2006), body weight gain (Hong et al., 2007) and plasma triglyceride concentration (Tsimihodimos et al., 2004) in MetS and NAFLD patients, metformin attenuates the production of glucose in type II diabetic patients (Rector et al., 2008). Fenofibrate, an anti-hyperlipidaemic, is a peroxisome proliferator-activated receptor-α (PPAR-α) agonist (Lee et al., 2013). Peroxisome proliferator-activated receptor-α triggers lipid oxidation in tissues by upregulation oxidative genes and enzymes (Ibarra-Lara et al., 2016).

The obstacles associated with the use of synthetic pharmaceutical drugs are that they are expensive, inaccessible, mono-therapeutic and can elicit deleterious side effects (Strang et al., 2012; Parasuraman, 2018). As a consequence, the majority of the global society favours the use of plant-derived ethnomedicinal extracts and phytochemicals as an intervention for metabolic diseases as they are affordable, accessible and perceived to be safer (Alves and Rosa, 2007). Furthermore, plant-derived ethnomedicinal extracts and phytochemicals typically possess multiple-therapeutic activities (Shori, 2015; Gopalan, 2017). Thus, ethnomedicines,

such as ginger, are appropriate for preventing and or managing multifactorial metabolic disorders (Tabatabaei-Malazy et al., 2015; Oliveira et al., 2019).

## 2.8 Ginger

Ginger, *Zingiber officinale*, is a flowering plant (Jude, 2016). Its rhizomes are used as a condiment worldwide (King, 2012) and for medicinal purposes (Khodaie and Sadeghpoor, 2015). In ancient therapeutic practice, ginger is reported to have been used to treat rheumatoid arthritis, neurodegenerative diseases, inflammation and asthma (Rees and Alcolado, 2005). Presently, ginger is used as an ethnomedicine to treat and or manage systemic components of MetS such as diabetes mellitus, NAFLD and cardiovascular diseases. The main phytochemicals that confer medicinal activities to ginger are gingerols, shogaols and zingerone (Ogunyinka et al., 2015). Gingerols are responsible for ginger's pungent aroma (Momin et al., 2015). Although gingerols possess antioxidant, anti-diabetic, anticancer, antimicrobial and anti-inflammatory, they are highly unstable compounds (Andriyani et al., 2015; Semwal et al., 2015). Gingerols are converted into shogaols, paradols and zingerone (Wei et al., 2017; You et al., 2019). Zingerone, used in the present study, is more stable than gingerols (Bilehal et al., 2011; Cui et al., 2018), and has been identified as key phytochemical in ginger that possess anti-inflammatory, antioxidant, lipolytic, antithrombotic and antidiabetic activities (Srinivasan, 2005; Cui et al., 2018).

## 2.9 Zingerone

Zingerone (vanillylacetone) is semi-water soluble and nontoxic alkaline compound found in ginger (Ahmad et al., 2015; Mir et al., 2018). It is formed from gingerols through retro-aldol reactions (Kumar et al., 2014; Yong-xin1 et al., 2012). Zingerone is used as an additive flavouring in food (Svetaz et al., 2014). Previous research reports that zingerone has antioxidant, anti-inflammatory, anticancer, immunostimulant, hepatoprotective, antidiabetic and anti-obesogenic activities (Srinivasan, 2005; Svetaz et al., 2014; Naidu et al., 2018). There are no studies that have reported any toxicity and or adverse effects following zingerone administration.

## 2.10 Pharmacological actions of zingerone

### *2.10.1 Antidiabetic*

The administration of 10 mg/kg zingerone normalised blood glucose concentration in STZ-diabetic rats (Jothi et al, 2017). Following histopathological analyses of the pancreas, Jothi et al. (2017) proposed that zingerone preserves and improves the functionality of pancreatic β-cells. Similarly, Anwer et al. (2019) recently reported that oral administration of 50-100 mg/kg zingerone mitigates serum insulin concentration and reduces serum glucose concentration in nicotinamide and streptozotocin treated rats. A study by Ahmad et al. (2018) showed that the intraperitoneal administration of 100 mg/kg zingerone to alloxan-induced diabetic rats improves serum insulin concentration. Zingerone exerts its anti-hyperglycaemic activity by improving insulin concentration. Moreover, Ahmad et al. (2018) further proposed that zingerone exerts its antidiabetic effects through its anti-inflammatory action. Research by Cui et al. (2018) revealed that 50 mg/kg zingerone administration attenuates diabetic nephropathy in db/db mice. Zingerone exerted it antidiabetic effects by downregulating nicotinamide adenine dinucleotide phosphate oxidase 4 against hyperglycaemic cytotoxicity and decreased serum insulin high concentration (Cui et al., 2018).

### *2.10.2 Anti-obesogenic*

A study by Mani et al. (2017) showed that the oral administration of 20 mg/kg zingerone protects rats against ethanol-induced obesity via modulating energy expenditure, stimulation of fatty acid oxidation and triggering adipocyte apoptosis. The oral administration of 100 mg/kg zingerone for 8 weeks reversed high-fructose diet-induced visceral obesity and dyslipidaemia (Narayanan et al., 2016). Narayanan et al. (2016) reported that zingerone exerted by its anti-obesogenic effect by upregulating hormone-sensitive lipase which consequently increases lipolysis. Kumar et al. (2014) reported that the intake of zingerone in feed reduced body fat in high-fat diet-fed rats, through enhanced norepinephrine mediated lipolysis in the adipocytes (Pulbutr et al., 2011).

The oral administration of 50-100 mg/kg zingerone improves lipid concentration in streptozotocin-diabetic rats by reducing TG, LDL and VLDL cholesterols synthesis (Anwer et al., 2019). Similarly, in another study by Hemalatha and Prince, (2015), it was found that zingerone prevents hyperlipidaemia in isoproterenol-induced myocardial infarcted rats. Furthermore, pre-treatment with 6 mg/kg zingerone for 14 days prevented dyslipidaemia via stimulation of anti-peroxidative pathways in isoproterenol-induced myocardial infarcted rats

(Hemalatha and Prince, 2015). El-Bassossy et al. (2017) reported that the oral administration of 20 mg/kg body zingerone for 7 weeks increased serum adiponectin concentration in streptozotocin-induced diabetic rats. Obesity is associated with reduced adiponectin (Hara et al., 2003), therefore zingerone exerted its anti-obesogenic activity by upregulating adiponectin expression and secretion. Moreover, zingerone has been reported to suppress inflammatory responses by inhibiting macrophage accumulation in mesenteric adipose tissue isolated from obese mice fed a high-fat diet (Woo et al., 2007). Jesudoss et al. (2017); Woo et al. (2007) reported that zingerone exudes its anti-obesogenic activity in mice through the inhibition of monocyte chemoattractant protein-1, released from adipocytes in high-fat fed-diet mice.

### *2.10.3 Antioxidant*

El-Bassossy et al. (2017) revealed that the oral administration of 20 mg/kg zingerone exerts its antioxidant activity by upregulating catalase activity in STZ-diabetic rats. A study by Hemalatha and Prince, (2015) reported that the oral administration of 6 mg/kg zingerone for 14 days increased the plasma vitamin C, vitamin E, and glutathione concentration in isoproterenol-induced myocardial infarcted rats. Zingerone exerted these antioxidant effects by neutralizing free radicals (Hemalatha and Prince, 2015). The oral administration of 20 mg/kg zingerone reduced the production of reactive oxygen species (ROS) and lipid peroxidation by-products and increased the antioxidant enzyme activity in ethanol-induced hepatotoxicity male rats (Mani et al., 2016). Orchard et al. (2005) reported that orally administering 25 or 50 mg/kg of zingerone reduces lipid peroxidation in the jejunum of cisplatin-induced oxidative damage adult male rats. *In vitro*, zingerone is reported to directly stimulate peroxynitrite scavenging activity and inhibit superoxide and nitric oxide radical formation (Shin et al., 2005). A study by Rao et al. (2009) reported that the administration of 10–100 mg/kg zingerone protects mice against whole-body gamma radiation exposure. Zingerone exerted its antioxidant activity through scavenging radiation-induced free radicals and by increasing the number of spleen colonies.

### *2.10.4 Anti-inflammatory*

The oral administration of 25 mg/kg zingerone attenuates cisplatin-induced ovarian and uterine toxicity in adult rats by inhibiting inflammation (Kaygusuzoglu et al., 2018). Similarly, a study done by Lokender Kumar et al. (2014) reported that the administration of zingerone decreases the expression of TNF-α in a peritonitis mice model. Additionally, Song et al. (2016) showed that intraperitoneal administration of zingerone at 10, 20 and 40 mg/kg protects adult rats

against LPS-induced inflammation by suppressing TNF-α and IL-6 production. The administration of 2 and 8 mg/kg of zingerone has been demonstrated to increase PPARs activity and suppress pro-inflammatory transcription nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*κ*B) expression in normal male rats (Chung et al., 2009). According to (Chung et al., 2009; Hsiang et al., 2013) zingerone exerts its anti-inflammatory action by increasing hepatocyte Nuclear Factor 4 (HNF-4) and PPARs activity while suppressing NF-*κ*B expression. A study done by Hsiang et al. (2013) reported that intrarectal administration of zingerone protects mice against 2,4,6- trinitrobenzene sulphonic acid (TNBS)-induced inflammation by decreasing NF-$_k$B expression and IL-1ß production. Moreover, *in vitro* studies revealed that zingerone interferes with MAPK signalling pathway and thus inhibits age-dependent pro-inflammatory NF-κB expression (Abbasi et al., 2015). El-Bassossy et al. (2017) reported that the administration of 20 mg/kg zingerone decreases cardiac transforming growth factor beta1 (TGFβ1) expression in STZ-diabetic rats.

Research evidence indicates that obesity is characterized by chronic low-grade inflammation (Ohashi et al., 2014), which leads to the development of metabolic complications including NAFLD, MetS and T-II-DM (Kolb and Mandrup-Poulsen, 2010). Thus, the anti-inflammatory activities of zingerone could be used to prevent obesity-related inflammation and the subsequent complications.

### *2.10.5 Anticancer*

Studies have revealed that intraperitoneal administration of zingerone at 10 mg/kg exudes anti-cancerous activities in mice via suppressing tumour progression, angiogenesis and the secretion of matrix metalloproteinases from Renca cells (Rahimlou et al., 2016).

Moreover, Vinothkumar et al. (2014) reported that the oral administration of 10, 20, 40 mg/kg zingerone reduces tumour incidences and aberrant crypt foci formation in a colon carcinogenesis male rat model induced with colon carcinogenesis. Studies have reported that, in mice, the intraperitoneal administration of 10 mg/kg zingerone suppresses tumour growth and progression through mitotic arrest and via the stimulation of tumour apoptosis (Choi et al., 2018; Gan et al., 2019). Narayanan et al. (2016) reported that the intragastric administration of 100 mg/kg zingerone improved liver function and reduced hepatic fibrosis and micro-vesicular steatosis formation in high-fructose diet-fed adult male rats.

Obesity, NAFLD and MetS are associated with increased risk of developing cancer (Bianchini et al., 2002). Therefore, the anti-cancerous effects of zingerone can be used to combat metabolic-related cancers.

### *2.10.6 Hepatoprotective effect*

The oral administration of 100-50 mg/kg zingerone protects male rats against alloxan-induced liver damage by increasing hepatic antioxidant activity (Ahmad et al., 2018). Likewise, Lee et al. (2018) showed that the 0.72 mg/kg intravenous injection of zingerone protects against lipopolysaccharide-induced hepatic damage in adult male rats. The oral administration of 10 mg/kg zingerone prevented hepatic lipid peroxidation, inflammation and collagen formation in tetrachloride intoxicated rats. Moreover, zingerone was shown to possess hepatoprotective and anti-fibrotic effects against dimethylnitrosamine-induced liver injury (Cheong et al., 2016).

Mani et al. (2016) reported that the oral administration of 10, 20, 40 mg/kg zingerone ameliorates alcohol-induced hepatotoxicity in adult male rats. A study by Mir et al. (2018), showed that the oral administration of zingerone at the dose of 50 mg/kg improves hepatic antioxidant status and restores plasma ALT and AST activity in cyclophosphamide-induced hepatotoxicity adult rats. Zingerone administered at 100 mg/kg attenuated hepatic micro- and macro-vesicular steatosis, inflammation and sinusoidal fibrosis in adult male rats fed a high-fructose diet (Narayanan et al., 2016). Zingerone exerts its hepatoprotective activities by scavenging free radicals, increasing antioxidant activity and by suppressing the production of inflammatory mediators in the liver.

# Chapter Three: Materials and Methods

### 3.1 Source of zingerone

Food grade (96% purity) zingerone was procured from Sigma-Aldrich (Louis, Missouri, United States).

### 3.2 Ethical clearance

The experiment was conducted at the Central Animal Services (CAS) unit at the Faculty of Health Sciences, University of the Witwatersrand. All experimental procedures adhered to the international principles on the care and use of laboratory animals (National Research Council, 2010) guidelines and were approved by the Animal Ethics Screening Committee (AESC number: 2017/010/71/B). All efforts were made to minimize the suffering of animals. The ethical clearance certificate and ethical modification and extension are included in the appendices (Appendices 1 and 2) of this dissertation.

### 3.3 Animals: feeding and housing

In this study, a total of 77 (38 males and 39 females), 21-day-old Sprague-Dawley rats were obtained from the Central Animal Services, University of the Witwatersrand. The rats were acclimatised to handling and weighing for three days. Throughout the experiment, the rats had *ad libitum* access to standard rat chow and drinking fluid. The drinking fluid of each rat was replenished twice a week. The rats were housed at the Central Animal Services (CAS) in individual Perspex cages that were lined with wood shavings for bedding. Room temperature was maintained at 24 ± 2 ºC with a 12-hour light and dark cycle (lights on between 7 am-7 pm). The rat chow used in this study was procured from Rodent breeder, (LabChef Nutrition hub, Stellenbosch South Africa), [(crude protein 240g/kg, crude fibre 45g/kg, crude oils and fats 53g/kg, calcium 14g/kg and phosphorous 8g/kg)].

### 3.4 Treatment regimen doses and drug vehicle medium preparation

A 20% (w/v) fructose solution was used in the study to induce metabolic dysfunction in the rats. The fructose solution was prepared using genetically modified foods (GMO) free fructose (Nature's choice, South Africa). According to the manufacturer's product information label, the

nutritional composition of the fructose was energy1680 kJ, protein 0g, carbohydrates 98.2g, fat 0g, fibre 0g and sodium12mg. The fructose solution was prepared by dissolving 200 g of fructose in 500 mL of warm water and making up the solution to 1000 mL. Red and blue food colourant (Robertsons Food Colouring, Libstar Operations (pty) Ltd, South Africa) were added to the plain water and fructose solution, respectively, for easy identification of the drinking fluids. Gelatine cubes were used as a vehicle of delivery of the treatments and were prepared as previously described by (Kamerman et al., 2004). Briefly, the gelatine was prepared by mixing 8 g of Sheridans, clear, unflavoured gelatine [Retailer Brands (Pty) Ltd], 8 g of Hulett brown sugar (Tongaat Hulett, KwaZulu-Natal, South Africa Ltd), 2g Beefy Bovril (Unilever, Johannesburg, South Africa) and 100 mL of boiling water. The treatments (fenofibrate and or zingerone) were then mixed with the gelatine after cooling. Thereafter, the gelatine containing treatments were placed in a refrigerator and allowed to solidify and form cubes. Prior to the administration of fenofibrate and or zingerone in gelatine cubes, the rats were habituated to eating plain gelatine cubes. The technique of using gelatine cubes to administer drugs orally to was developed, and successfully used (Karmeman et al., 2004) in place of oral gavage which stresses animals..

### 3.5 Experimental design

Following two days of habituation, the seventy-seven, 21-day-old Sprague-Dawley rats were randomly allocated and administered one of the following four treatment regimens for 12 weeks:
regimen I (negative control; 8 males and 10 females): were fed standard rat chow (SR) + plain water (PW) + plain gelatine cube (PC).
regimen II (positive high-fructose diet control ; 10 males and 10 females): were fed SR + 20% (w/v) fructose solution (FS) + PC.
regimen III (positive control intervention; 10 males and 9 females): were fed SR + FS + 100 mg/kg/day of fenofibrate in gelatine cube (Feno).
regimen IV (zingerone intervention ; 10 males and 10 females): SR+ FS + 20 mg/kg/day of zingerone in gelatine cube (Zing).
The rats had *ad libitum* access to standard rat chow and were maintained to their respective treatment regimens for 12 weeks.

Throughout the experiment, the rats were weighed twice a week using an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg). The rats' body weights were used to monitor growth performance and to maintain constant doses of the zingerone and fenofibrate relative to body weight for the period of 12 weeks.

The fenofibrate and zingerone doses used in the current study are similar to those used by Kim et al. (2010) and; Vinothkumar et al. (2014).

### 3.6 Oral glucose tolerance test

Following 12-weeks of treatments, the rats were fasted overnight but allowed access to plain water to drink. Thereafter the rats' fasting blood glucose concentration was determined using a calibrated Contour plus glucose meter [Contour plus, blood glucose monitoring system, Bayer (Pty) Ltd., Praha, Czech Republic]. Immediately thereafter the rats were orally gavaged with 2g/kg of 50% w/v D-(+)-sterile glucose solution [Dextrose glucose, Merck Chemicals (Pty) Ltd, Johannesburg, South Africa] (Kannappan and Anuradha, 2010). Blood glucose concentration was then determined at 15, 30, 60- and 120-minutes post-gavage. The glucose concentrations were determined using a drop of blood obtained from the tail vein via pinprick (Loxham et al., 2007), after being sterilized with alcohol prior to pinpricking.

### 3.7 Terminal activities

Following the oral glucose tolerance tests, the rats were returned to their respective treatments for 48 hours to allow them to recover prior to being fasted overnight and euthanised on postnatal day 109. The rats' terminal body weights and fasting blood glucose concentration were determined using an electronic balance (Presica 310 M, Laser, Johannesburg South Africa) and a calibrated Contour plus glucose meter, respectively. Thereafter each rat was euthanised by intraperitoneal injection with an overdose of 200mg/kg of sodium pentobarbitone (Euthanaze®, Centaur labs, Johannesburg, South Africa). Following euthanisation, the rats were cut open by a midline incision and blood was collected via cardiac puncture using 21G needle and 10 mL syringe and transferred into 10mL heparinised blood collection tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The heparinised blood samples were

centrifuged at 5500×*g* for 15-min (Hermle Centrifuge Z 230 A, Berthold Hermle AG, German) to produce plasma. The plasma was stored in microtubes (Eppendorf, Hamburg, Germany) at 20°C for further assay analyses. The visceral fat and livers of each rat were carefully dissected out. Samples of the livers were then collected and stored at -20°C for the determination of total liver content. Liver histology samples were collected and preserved in 10% buffered formalin until analysis. The eviscerated rat carcasses were weighed on an electronic balance (Presica 310 M, Precision Instruments, Johannesburg, South Africa) to determine empty carcass weights. The carcasses were stored in a freezer for the determination of long bone indices.

### 3.8 Determination of long bone indices

The femoral attachment of left hind leg to the pelvis was gently dissected using a pair of scissors and scalpel blade, from each of the carcasses, defleshed and disarticulated from the tibiae. The tibiae were dried in an oven (Salvis ®, Salvis Lab, Switzerland) at 50 °C for 5 days until constant weights were reached. An electronic balance (Presica 310M Laser, Johannesburg, South Africa) was then used to determine the weight of the tibiae. The lengths of the tibiae were then measured using digital callipers [Major Tech (Pty) Ltd, KTV 150 digital calliper, Elandsfontein, South Africa]. Bone weight to length ratio was then estimated using the equation:bone mass to bone length ratio = dry mass of bone (mg) /bone length (mm) (Seedor et al., 1991).

### 3.9 Blood measurements

#### *3.9.1 Determination of plasma insulin and adiponectin*

The plasma insulin and adiponectin concentrations were determined using rat antibody-specific insulin and adiponectin enzyme-linked immunosorbent assays (ELISA; Elabscience® ELİSA Kit) according to the manufacturer's instructions, respectively. While the sensitivity values for insulin and adiponectin were 0.19 ng/ml and 28.13 pg/ml, respectively, the detection ranges were 0.31-20 ng/ml and 46.88-3000 pg/ml, respectively. All the reagents and plates were supplied in the kit. The absorbencies were read at 450 nm using a microplate reader (Multiskan Ascent, Lab system, model nº 354, Helsinki, Finland). The standard curves were constructed using respective

calibrator concentrations and the concentrations of insulin and adiponectin in the samples were determined using the constructed standard curves.

#### *3.9.1.1 Computation of the HOMA-IR*

Fasting whole-body insulin sensitivity was estimated using the homoeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al., 1985)

HOMA-IR = [fasting insulin (mU/L) × fasting glucose (mg/dL) / 22.5].

### *3.9.2 Determination of plasma total cholesterol and triglyceride*

The triglyceride assay kit (Elabscience Biotechnology Inc., Single Reagent GPO-PAP, USA) and total cholesterol assay kit (Elabscience Biotechnology Inc., Single reagent, GPO-PAP, USA) and TG Assay Kit (Elabscience Biotechonology Inc.,Single reagent, GPO-PAP, USA) enzyme-based assay kits were used to determine plasma total cholesterol and triglyceride concentrations. Plasma total cholesterol and triglyceride concentration were determined using single reagent cholesterol oxidase p-aminophenol (COD-PAP) and single reagent glycerol-3-phosphate oxidase-phenol + aminophenazone (GPO-PAP) methods, respectively.

### *3.9.3 Determination of aspartate aminotransferase*

Aspartate aminotransferase was 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 plasma sample was allowed to thaw to room temperature, and then gently inverted to mix the contents and then placed into the analyser machine which automatically drew up 300 μL of the plasma sample. Thereafter, the analyser automatically loaded 10 μL of plasma sample onto aspartate aminotransferase analysis cassette inside the analyser machine. The sample was analysed, and the value of the analysed sample was displayed on the colorimetric-based clinical chemistry analyser.

## 3.10 Determination of liver lipid

Liver lipid analyses were carried out at the Agricultural Research Council (Irene Analytical Services Laboratory) using the Soxhlet ether extraction method (Association of Official

Agricultural Chemists, Official Method 920.39). Briefly, the liver samples were freeze-dried (Virtis Sentry 2.0, New York, NY, USA) and milled to a powder. Three grams of the dried liver sample was placed in a soxhlet thimble. The thimble was then placed in a pre-weighed flask containing 60 mL 80% petroleum ether. The flasks, with the thimble, were placed under the Soxtec extraction unit and the samples were extracted for 30 minutes at 90°C. The flasks containing the extracted lipids (oil) dried in the oven and then cooled in a desiccator for 30 minutes. The extracted lipids from the liver samples were then calculated using the formula:

% lipid = [(mass of flask with fat – the mass of flask) ÷ (mass of sample)] x 100

### 3.11 Liver histology

The preserved liver samples were routinely processed, embedded in paraffin wax, sectioned to a thickness of 5 μm. Thereafter the slide sections were stained with haematoxylin and eosin and covered with a glass coverslip. The liver slides were then viewed under a microscope at a magnification of 400 X using an eyepiece micrometre (Reichert®, Austria) for assessment of liver steatosis and scored using the NAFLD Activity Score (NAS) criteria: *steatosis scoring 0: <5%; 1: 5–33%; 2: 34–66%; 3: >66%; foci of lobular inflammation scoring 0: none; 1: <2; 2: 2–4; 3: >4; hepatocellular ballooning scoring 0: none; 1: few ballooned cells; 2: many ballooned cells; total NAS (sum of values recorded for each criteria) < 2: not steatohepatitis; 3 and 4: uncertain; >5: probable or definite steatohepatitis* (Kleiner et al., 2005).

### 3.12 Statistical analysis

All data were analysed using Graph Pad Prism 8.0.2 (Graph-pad Software Inc., San Diego, USA). Parametric data are presented as mean ± SD. Weekly body weight and OGTT within-group data were analysed using a repeated measure analysis of variance (ANOVA). A one-way ANOVA followed by a Bonferroni *post hoc* test was used to analyse multiple-group parametric data. Multiple-group, non- parametric data were analysed using a Kruskal-Wallis test followed by Dunns *post hoc* test. Statistical significance was accepted when $P < 0.05$.

# Chapter Four: Results

### 4.1 Body weight and empty carcass weight

Figures 4.1 and 4.2 show the induction and terminal body weights of the male and female rats, respectively. There were statistical differences in the induction weights of the male rats across treatment regimens (Figure 4.1). Rats that were fed the high-fructose diet in combination with fenofibrate (FS + Feno) had significantly reduced ($P < 0.0001$) terminal body weights compared to control (PW + PC) counterparts (Figure 4.1). The induction and terminal weights of female rats were similar across all treatment regimens (Figure 4.2). Male and female rats grew significantly ($P < 0.0001$) from induction to termination (Figures 4.1 and 4.2).

The empty carcass weights of the male and female rats are shown in Figures 4.3 and 4.4, respectively. Empty carcass weights of the male rats fed the high-fructose diet in combination with fenofibrate (FS + Feno) were significantly lighter ($P < 0.0001$) compared to their male counterparts who received PW + PC or FS + Zing (Figures 4.3). The empty carcass weights of the female rats were similar across treatments regimens (Figures 4.4).

### 4.2 Tibiae weights , lengths and weight to length ratio

Tables 4.1and 4.2 show the tibiae weights, lengths and weight to length ratios of male and female rats, respectively. The tibiae weights, lengths and weight to length ratios of the male and female rats were similar ($P > 0.05$) across treatment regimens (Table 4.1 and 4.2).

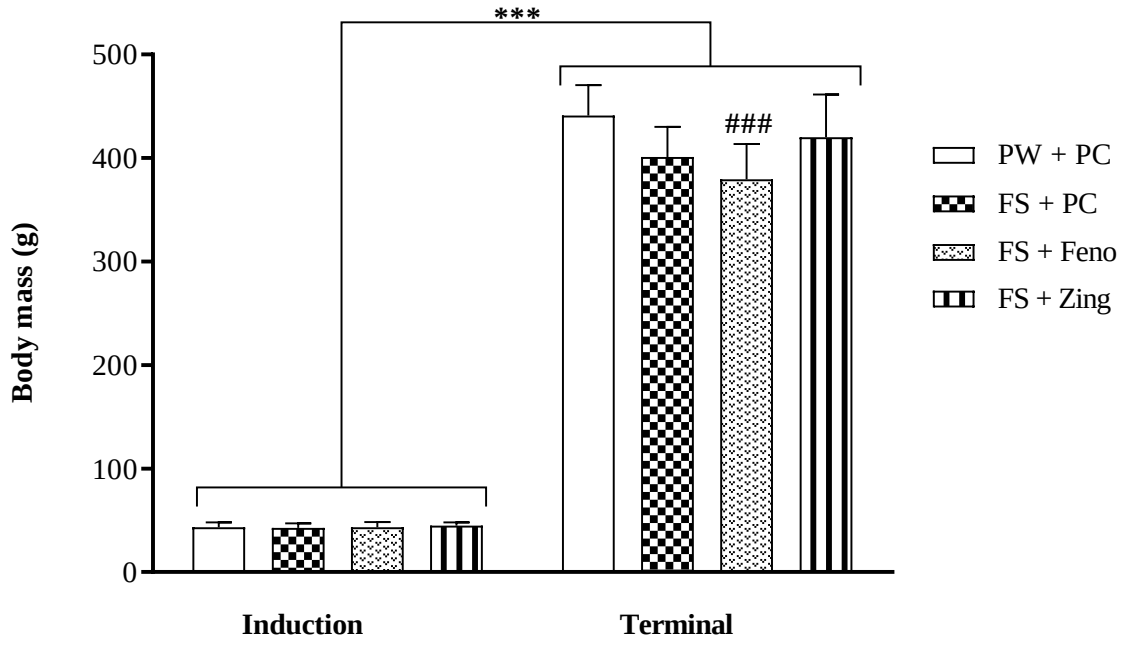



**Figure 4.1: The induction and terminal body weights of the male rats.**

*** P < 0.0001 when induction was compared to terminal; ### P < 0.0001when FS + Feno was compared to PW + PC. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

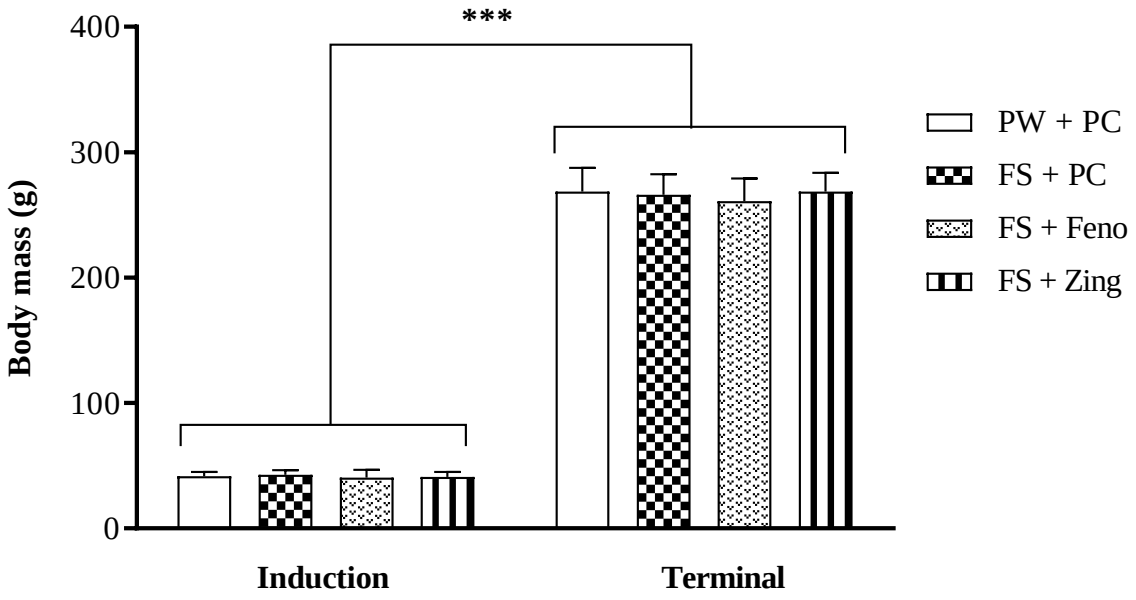



**Figure 4.2: The induction and terminal body weights of the female rats.**

*** P < 0.0001 when induction was compared to terminal. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

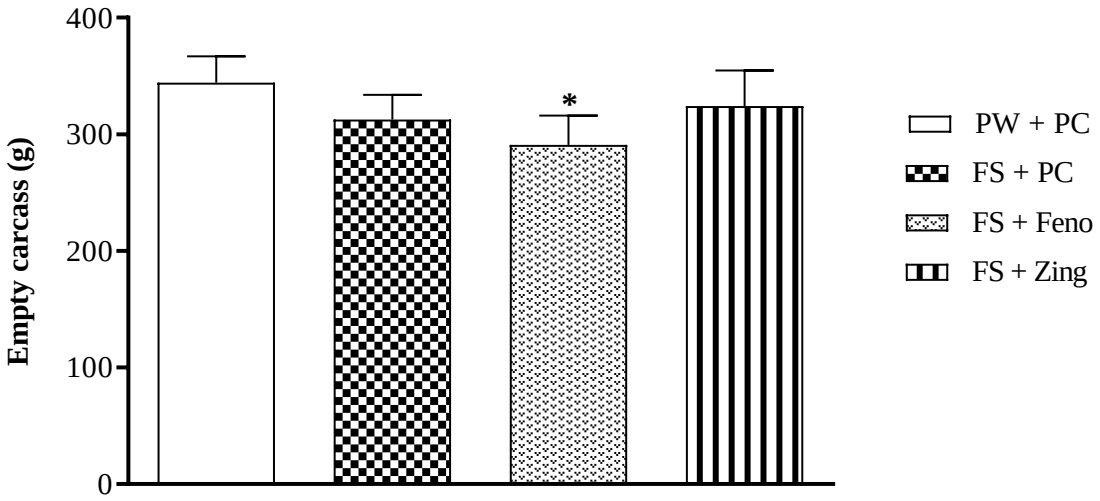



**Figure 4.3: Effect of zingerone on empty carcass weight of male rats fed a high-fructose diet.**

*P < 0.05 when compared to PW + PC and FS + Zing. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 8-10 per treatment.

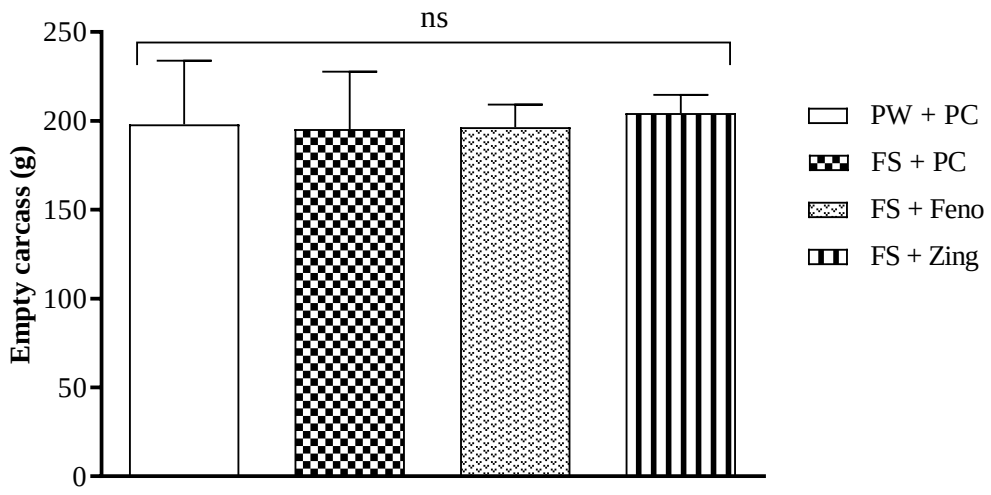



**Figure 4.4: Effect of zingerone on empty carcass weight of female rats fed a high-fructose diet.**

ns = not significant, P > 0.05. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

**Table 4.1: Effect of zingerone on tibiae lengths, weights and weight to length ratio of male rats fed a high-fructose diet.**

| Tibia | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Weight (mg) | 589.40 ± 63.05 | 561.7 ± 50.37 | 555.1 ± 61.85 | 616.6 ± 53.64 | ns |
| Length (mm) | 21.36 ± 1.40 | 20.82 ± 1.18 | 20.48 ± 0.94 | 21.17 ± 0.68 | ns |
| Weight:length (mg/mm) | 27.56 ± 1.87 | 26.96 ± 1.59 | 27.6 ± 2.26 | 29.09 ± 1.72 | ns |

ns = not significant, $P > 0.05$. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 8-10 per treatment.

**Table 4.2: Effect of zingerone on tibiae lengths, weights and weight to length ratio in high-fructose diet-fed female rats**

| Tibia | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Weight (mg) | 488.6 ± 77.72 | 484.8 ± 16.59 | 439.0 ± 41.58 | 477.8 ± 46.54 | ns |
| Length (mm) | 19.68 ± 0.94 | 19.54 ± 0.46 | 18.98 ± 0.76 | 19.07 ± 1.01 | ns |
| Weight:length (mg/mm) | 24.73 ± 3.09 | 24.81 ± 0.6742 | 23.11 ± 1.728 | 25.02 ± 1.62 | ns |

ns = not significant, $P > 0.05$. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

### 4.3 Oral glucose tolerance test

The male and female rats' blood glucose concentrations at basal (0 minute) and 15, 30, 60- and 120-minutes post-gavage with 50% glucose solution at 2 g/kg body weight are shown in Figures 4.5 and 4.6, respectively. Basal blood glucose concentrations of male rats were similar across treatment regimens (Figure 4.5). Blood glucose concentrations of male rats across treatment regimens peaked ($P < 0.0001$) 15 minutes post-gavage (Figure 4.5). The blood glucose concentration of male rats given FS + Zing returned to basal concentration 30 minutes post-gavage (Figure 4.5). While the blood glucose concentration of PW + PC and FS + PC male rats returned to basal concentration at 60 minutes post-gavage, the blood glucose concentration of male rats given FS + Feno returned to basal concentration 120 minutes post-gavage (Figure 4.5).

In female rats, the basal blood glucose concentrations were similar across treatment regimens (Figure 4.6). However, blood glucose concentrations peaked ($P < 0.05$) 15 minutes post-gavage across female treatment regimens (Figure 4.6). Blood glucose concentrations of treatment regimens FS + Zing female rats returned to basal at 30 minutes post-gavage (Figure 4.6). The blood glucose concentrations of female rats given PW + PC, FS + PC and FS + Feno returned to basal concentrations 60 minutes post-gavage (Figure 4.6).

Figures 4.7 and 4.8 shows the area under the curve calculated from oral glucose tolerance test results. The area under the curve of oral glucose tolerance test of rats given FS + Feno was significantly ($P < 0.0001$) higher compared to the area under the curve of oral glucose tolerance test from rats given other treatment regimens. Importantly, other treatment regimens had similar ($P > 0.05$) area under the curve of the oral glucose tolerance test (Figure 4.7 and 4.8).

### 4.4 Blood parameters

Tables 4.3 and 4.4, shows the effects of zingerone on plasma triglycerides and cholesterol of the male and female rats, respectively. The cholesterol and triglycerides concentration in the plasma were similar ($P > 0.05$) across all male treatment regimens (Table 4.3). In female rats, the high-fructose diet and zingerone had no effect on the plasma triglyceride and cholesterol concentrations (Table 4.4). Fenofibrate significantly increased ($P = 0.04$ compared to control) plasma triglyceride concentration (Table 4.4).

Tables 4.5 and 4.6, shows the effects of zingerone on blood glucose, plasma adiponectin, insulin, HOMA-IR and AST concentration of the male and female rats, respectively. The high-fructose diet and zingerone had no effect on blood glucose and plasma insulin concentrations as well as HOMA-IR of the male and female rats (Table 4.5 and 4.6). The blood glucose and plasma insulin concentrations as well as HOMA-IR of male rats that were administered fenofibrate were significantly increased compared to FS + PC (Table 4.5). Fenofibrate had no effect on the blood glucose and plasma insulin concentrations as well as HOMA-IR of female rats (Table 4.6). The treatments had no effect on plasma adiponectin concentration and AST activity of male and female rats (Tables 4.5 and 4.6).

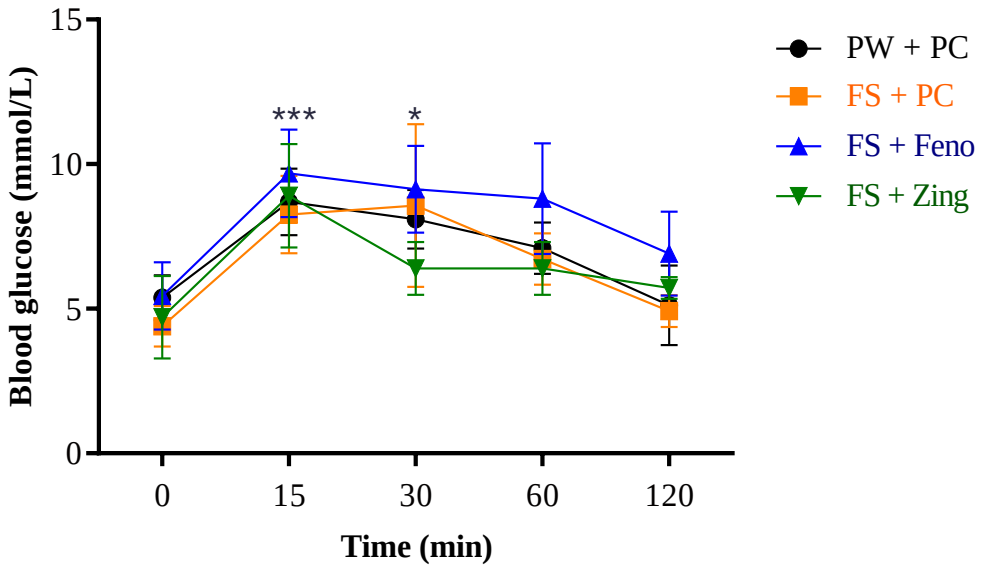



**Figure 4.5: Effect of zingerone on the ability to tolerate oral glucose in male rats fed a high-fructose diet.**

*** P < 0.0001 when all treatment regimens were compared to basal; *P < 0.05 when PW + PC, FS + PC and FS + Feno blood glucose concentration, 30 minutes post-gavage, were compared to basal. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 8 per treatment.

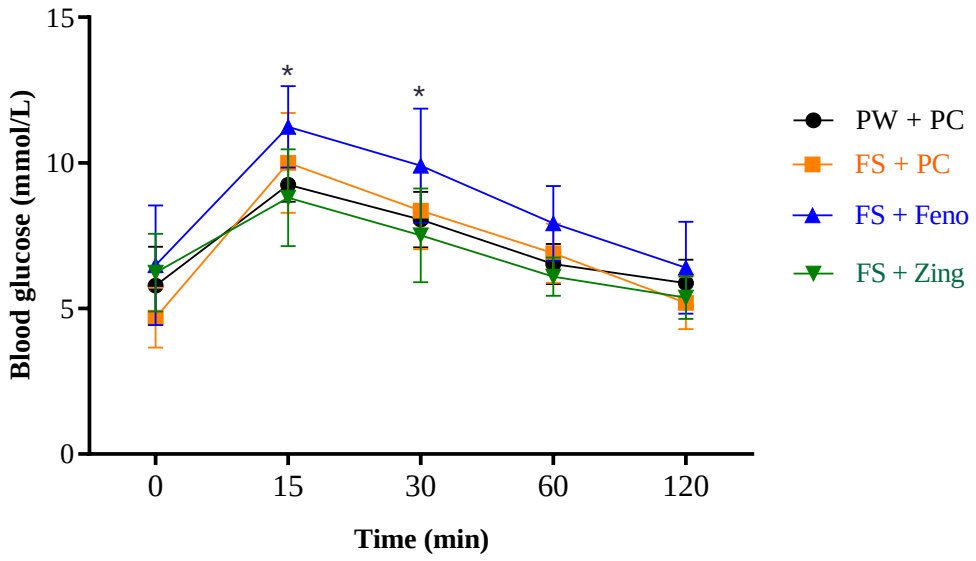



**Figure 4.6: Effect of zingerone on the ability to tolerate oral glucose in female rats fed a high-fructose diet.**

*P < 0.05 when all treatment regimens were compared to basal. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 8 per treatment.

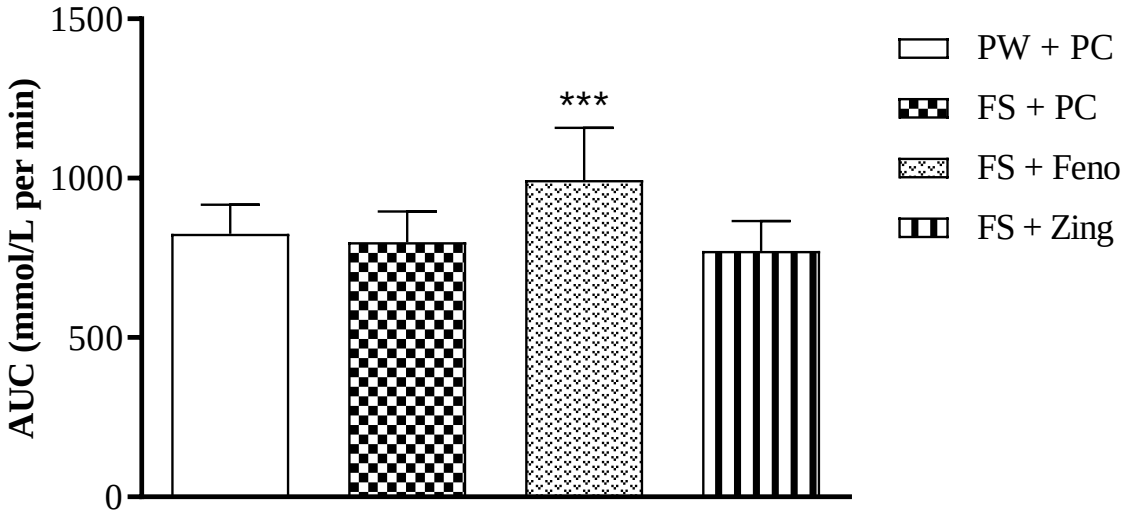



**Figure 4.7: Effect of zingerone on total area under the curve of oral glucose tolerance of male rats fed a high-fructose diet.**

*** P < 0.0001 when compared to all other treatment regimens. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. AUC = area under the curve. Data presented as mean ± SD; n = 8 per treatment.

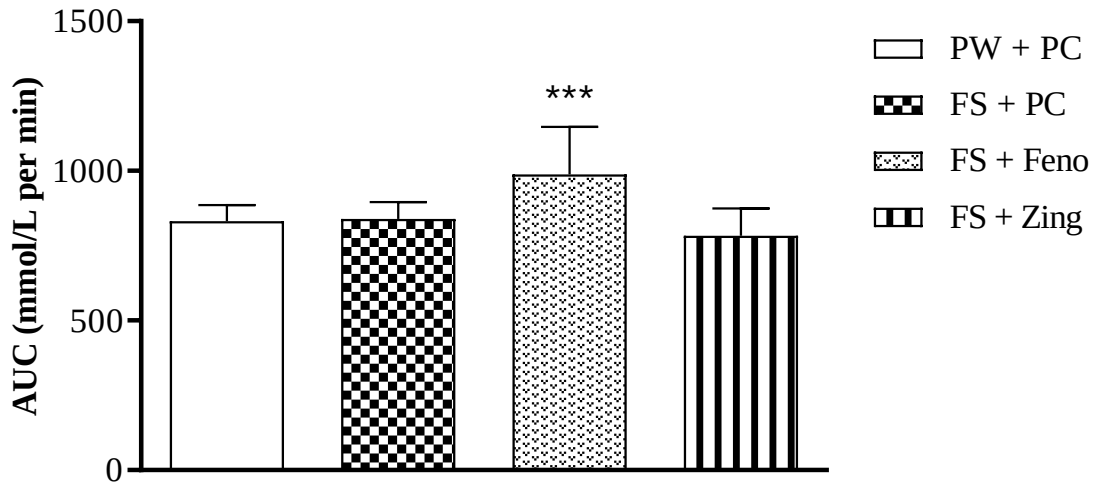



**Figure 4.8: Effect of zingerone on total area under the curve of oral glucose tolerance of female rats fed a high-fructose diet.**

*** $P < 0.0001$ when compared to all other treatment regimens. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. AUC = area under the curve. Data presented as mean ± SD; n = 8 per treatment.

**Table 4.3: Effect of zingerone on plasma triglyceride and cholesterol concentration of male rats fed a high-fructose diet**

| Blood parameters | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Triglycerides (mmol/L) | 0.68 ± 0.65 | 0.81 ± 0.37 | 0.85 ± 0.55 | 0.64 ± 0.48 | ns |
| Cholesterol (mmol/L) | 4.13 ± 1.73 | 5.58 ± 1.87 | 2.55 ± 1.55 | 12.01 ± 18.95 | ns |

ns = not significant, P > 0.05. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 8-10 per treatment.

**Table 4.4: Effect of zingerone on plasma triglyceride and cholesterol concentration of female rats fed a high-fructose diet**

| Blood parameters | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Triglycerides (mmol/L) | $0.34 \pm 0.33^{a}$ | $0.51 \pm 0.45^{ab}$ | $0.80 \pm 0.31^{b}$ | $0.44 \pm 0.31^{ab}$ | * |
| Cholesterol (mmol/L) | $5.62 \pm 2.86^{a}$ | $6.82 \pm 2.10^{a}$ | $5.84 \pm 1.28^{a}$ | $5.75 \pm 3.84^{a}$ | ns |

ns = not significant, *P < 0.05. [ab]Within row means with different superscripts are significantly different at P < 0.05. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

**Table 4.5: Effect of zingerone on blood glucose, insulin, adiponectin, HOMA-IR and AST concentration of male rats fed a high-fructose diet**

| Blood parameters | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Glucose (mmol/L) | 3.95 ± 0.40[a] | 3.71 ± 0.48[a] | 4.32 ± 0.43[b] | 3.79 ± 0.45[a] | * |
| Insulin (ng/mL) | 1.50 ± 0.54[a] | 1.28 ± 0.55[a] | 2.09 ± 0.85[b] | 1.96 ± 0.44[a] | * |
| Adiponectin (pg/mL) | 154.10 ±86.93[a] | 208.40 ± 197.0[a] | 175.60 ± 118.47[a] | 262.50 ± 108.70[a] | ns |
| HOMA-IR | 5.21 ± 2.02 [ab] | 3.75 ± 1.60 [a] | 7.11 ± 2.70[b] | 5.92 ± 1.42 [ab] | ** |
| AST (U/L) | 165.30 ± 52.86[a] | 118.30 ±38.19[a] | 167.50 ± 54.33[a] | 232.50 ± 246.60[a] | ns |

ns = not significant, **P < 0.01, *P < 0.05. [ab]Within row means with different superscripts are significantly different at P < 0.05. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 6-10 per treatment.

**Table 4.6: Effect of zingerone on blood glucose, plasma insulin, adiponectin, HOMA-IR and AST concentration of female rats fed a high-fructose diet**

| Blood parameters | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Glucose (mmol/L) | 3.86 ± 0.28 | 3.60 ± 0.40 | 4.49 ± 0.92 | 3.77 ± 0.38 | ns |
| Insulin (ng/mL) | 1.60 ± 0.45 | 1.53 ± 0.68 | 1.43 ± 0.80 | 1.60 ± 0.38 | ns |
| Adiponectin (pg/mL) | 216.20 ± 94.84 | 230.50 ± 116.20 | 227.80 ± 137.8 | 266.90 ± 127.80 | ns |
| HOMA-IR | 4.94 ± 1.33 | 4.40 ± 1.93 | 5.28 ± 3.57 | 4.81 ± 1.22 | ns |
| AST (U/L) | 107.50 ± 26.07 | 95.17 ± 23.50 | 188.30 ± 165.8 | 122.20 ± 47.57 | ns |

ns = not significant, $P > 0.05$. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 6-10 per treatment.

### 4.5 Histology

The representative pictures of the liver histology section (haematoxylin and eosin staining, 400 X magnifications) of male and female rats are shown in Figure 4.9 and 4.10, respectively. The high-fructose diet caused macro-vesicular hepatic steatosis in male rats (Figure 4.9) and micro-vesicular hepatic steatosis in female rats (Figure 4.10). Zingerone and fenofibrate prevented the hepatic steatosis in the high-fructose diet-fed rats (Figures 4.9 and 4.10).

### 4.6 Viscera morphometry

Tables 4.7 and 4.8, below shows the male and female rats' absolute and relative (to tibia length and body weight) liver and visceral fat weights, respectively. Fenofibrate significantly increased ($P < 0.05$ compared to all treatments) absolute and relative liver weights of male and female rats (Tables 4.7 and 4.8). The high-fructose diet and fenofibrate had no effect on the visceral fat weight of the rats (Figures 4.7 and 4.8). Zingerone significantly increased ($P < 0.05$ compared to control) visceral fat weight relative to body weight in high-fructose diet-fed female rats but not in male rats (Tables 4.7 and Table 4.8).

### 4.7 Liver lipid content

Figures 4.11 and 4.12 shows liver lipid content, of the male and female rats, respectively. The high-fructose diet significantly increased ($P < 0.0001$) liver lipid content in male and female rats (Figures 4.11 and 4.12). Zingerone did not prevent the high-fructose diet-induced increase in liver lipid accretion in the rats (Figures 4.11 and 4.12). In male rats, fenofibrate prevented ($P < 0.0001$ compared to PC + PW and FS + PC) the high-fructose diet-induced increase in liver lipid accumulation (Figure 4.11). However, in female rats, fenofibrate attenuated the high-fructose diet-induced increased liver lipid accretion (Figure 4.12).

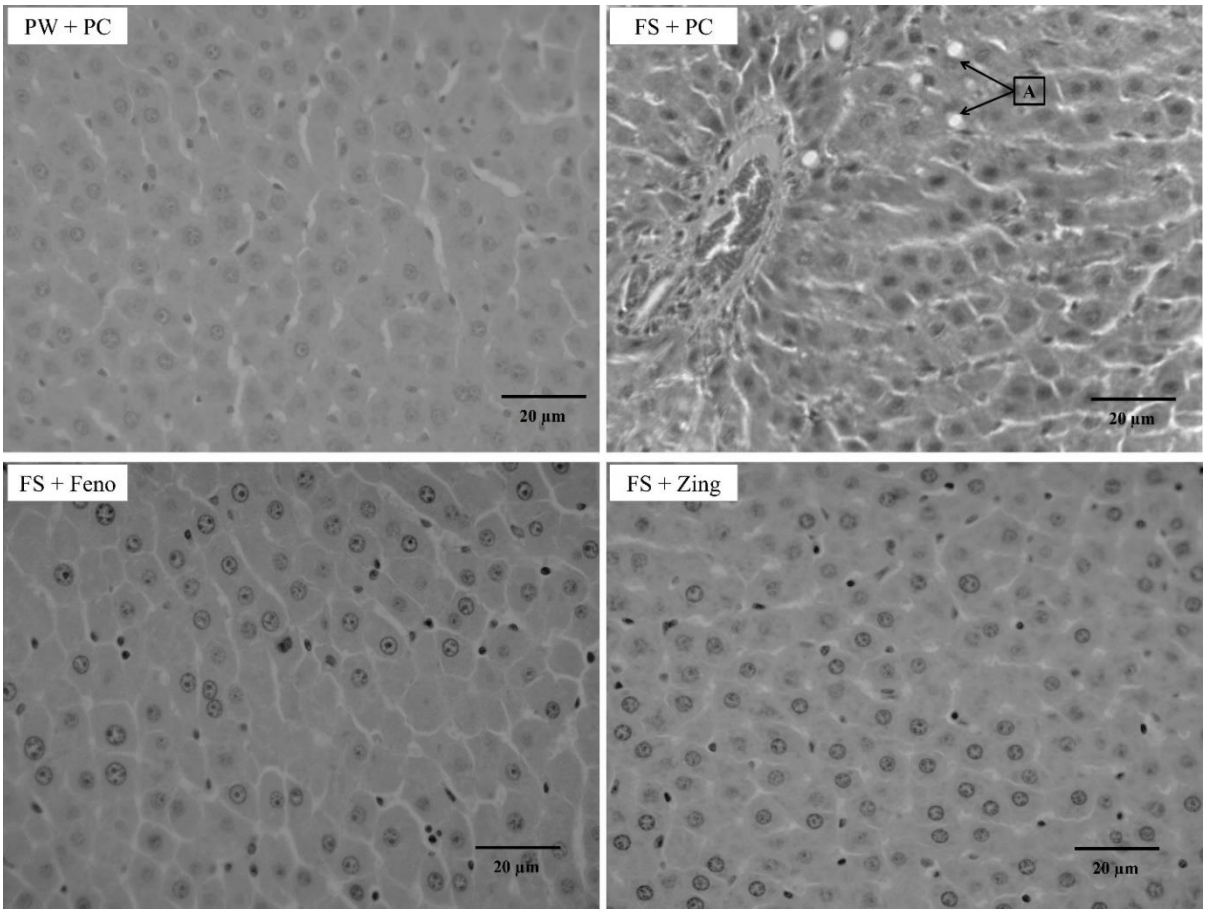



**Figure 4.9: Liver histology sections (H and E staining, 400 X magnifications) of male rats fed a high-fructose diet.**

Arrows: A indicates macro-vesicular steatosis. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube.

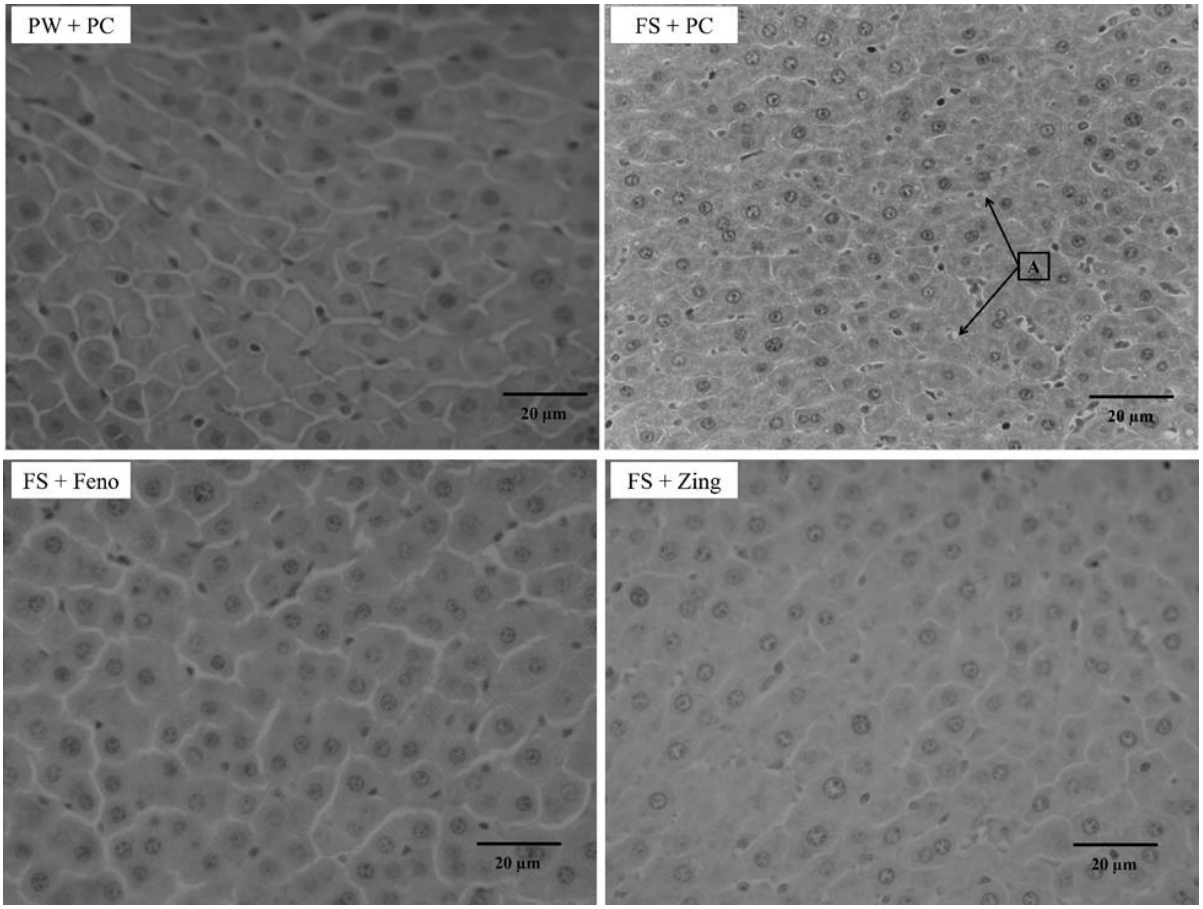



**Figure 4.10: Liver histology sections (H and E staining, 400 X magnifications) of female rats fed a high-fructose diet.**

Arrows: A shows macro-vesicular steatosis. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube.

**Table 4.7: Effect of zingerone on the liver and visceral fat absolute and relative weights in male rats fed a high fructose diet**

| Organ | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Liver (g) | 12.93 ± 2.20[a] | 12.26 ± 1.19[a] | 16.63 ± 1.93[b] | 13.96 ± 1.95[a] | * |
| Liver (%BW) | 2.92 ± 0.38[a] | 3.06 ± 0.24[a] | 4.38 ± 0.30[b] | 3.34 ± 0.46[a] | *** |
| Liver TLr (g/cm) | 6.02 ± 0.75[a] | 5.91 ± 0.69[a] | 8.13 ± 0.96[b] | 6.59 ± 0.81[a] | ** |
| VFP (g) | 11.21 ± 2.52 [a] | 11.67 ± 2.75[a] | 9.48 ± 3.72[a] | 12.66 ± 1.99[a] | ns |
| VFP (%BW) | 2.53 ± 0.50 [a] | 2.89 ± 0.54[a] | 2.45 ± 0.69[a] | 3.01 ± 0.31[a] | ns |
| VFP TLr (g/cm) | 5.52 ± 1.18 [a] | 5.65 ± 1.54[a] | 4.61 ± 1.69[a] | 5.97 ± 0.85[a] | ns |

ns = not significant, ***P < 0.0001; ** P < 0.01; *P < 0.05.[abc]Within row means with different superscripts are significantly different at P < 0.05. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube.VFP = Visceral fat pad; BW = Body weight; TLr = Relative tibia length. Data presented as mean ± SD; n = 8-10 per treatment.

**Table 4.8: Effect of zingerone on other visceral fat absolute and relative weights in female rats fed a high fructose diet**

| Organ | PW + PC | FS + PC | FS + Feno | FS + Zing | Significance |
|---|---|---|---|---|---|
| Liver (g) | $7.32 \pm 0.81^{a}$ | $7.74 \pm 0.64^{a}$ | $11.41 \pm 1.46^{b}$ | $7.94 \pm 0.80^{a}$ | *** |
| Liver (%BW) | $2.73 \pm 0.23^{a}$ | $2.91 \pm 0.19^{a}$ | $4.38 \pm 0.53^{b}$ | $2.96 \pm 0.27^{a}$ | * |
| Liver TLr (g/cm) | $3.72 \pm 0.40^{a}$ | $3.96 \pm 0.29^{a}$ | $6.03 \pm 0.89^{b}$ | $4.17 \pm 0.46^{a}$ | *** |
| VFP (g) | $12.85 \pm 2.88^{ab}$ | $14.20 \pm 2.83^{ab}$ | $11.79 \pm 2.96^{a}$ | $16.01 \pm 3.51^{b}$ | * |
| VFP (%BW) | $4.77 \pm 0.87^{a}$ | $5.32 \pm 0.88^{ab}$ | $4.47 \pm 0.86^{a}$ | $5.92 \pm 0.96^{b}$ | ** |
| VFP TLr (g/cm) | $6.53 \pm 1.43^{ab}$ | $7.26 \pm 1.37^{ab}$ | $6.19 \pm 1.45^{a}$ | $8.42 \pm 1.97^{b}$ | * |

ns = not significant, ***$P < 0.0001$; ** $P < 0.01$; * $P < 0.05$. $^{ab}$Within row means with different superscripts are significantly different at $P < 0.05$. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. VFP = Visceral fat pad; BW = Body weight; TLr = Relative tibia length. Data presented as mean ± SD; n = 9-10 per treatment.

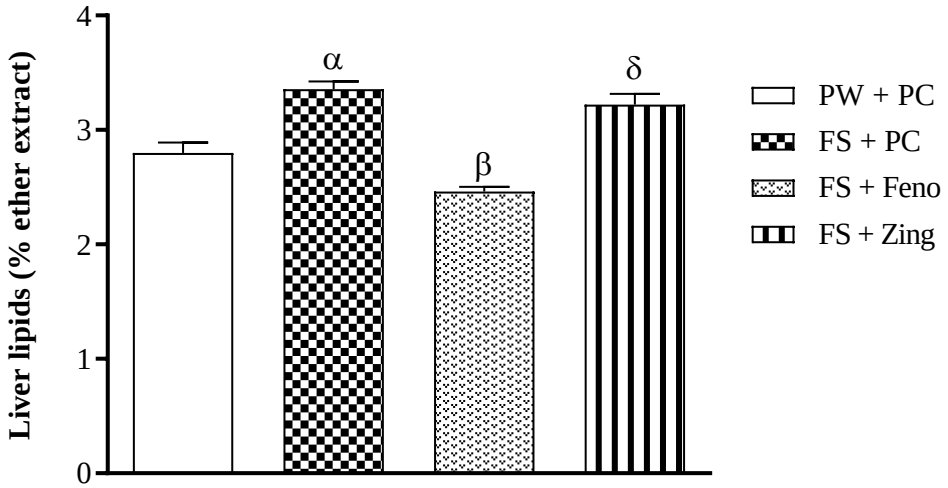



**Figure 4.11: Effect of zingerone on the hepatic lipid content of male rats fed a high-fructose diet.**

α, $P < 0.0001$ compared to PW + PC, FS + Feno; β, $P < 0.0.5$ compared to PW + PC, FS + Zing; δ, $P < 0.01$ compared to PW + PC. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

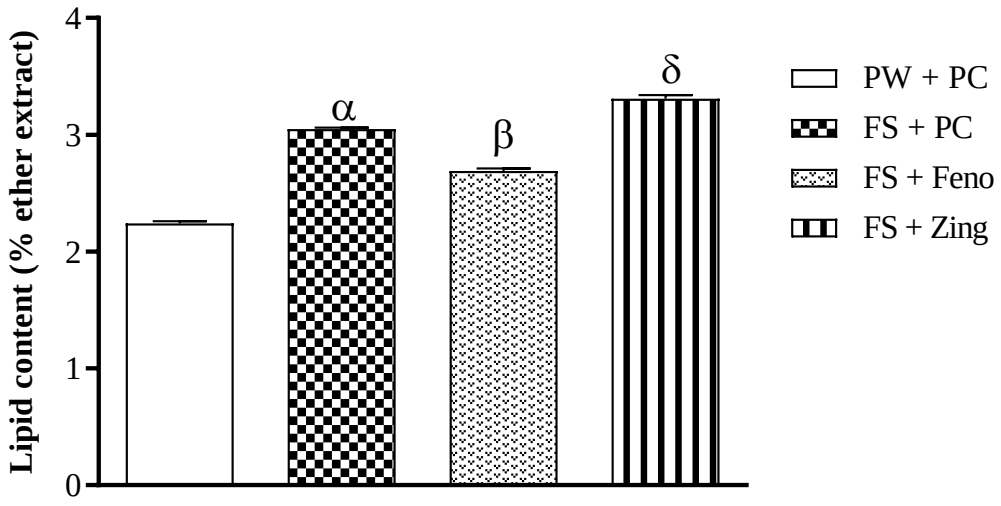



**Figure 4.12: Effect of zingerone on the hepatic lipid content of female rats fed a high-fructose diet.**

α, $P < 0.01$ compared to PW + PC, FS + Feno, FS + Zing; β, $P < 0.0001$ compared to PW + PC and FS + Zing; δ, $P < 0.0001$ compared to PW + PC. PW + PC = standard rat chow + plain water + plain gelatine cube; FS + PC = standard rat chow + 20% fructose solution + plain gelatine cube; FS + Feno = standard rat chow + 20% fructose solution + 100 mg/kg body weight per day fenofibrate in gelatine cube; FS + Zing = standard rat chow + 20% fructose solution + 20 mg/kg body weight per day zingerone in gelatine cube. Data presented as mean ± SD; n = 9-10 per treatment.

# Chapter Five: Discussion

My study sought to determine the effects of zingerone on growth performance, metabolic substrate (plasma glucose, triglycerides and total cholesterol) and liver lipid content as well as hormone (insulin and adiponectin) content, tolerance to an oral glucose load, liver function and micro-architecture in Sprague-Dawley rats fed a high-fructose diet. The high-fructose diet did not affect the rats' growth performance, tolerance and sensitivity to an oral glucose load, adiponectin concentration, liver mass and function as measured by plasma AST activity. It also did not cause visceral adiposity, hypercholesterolemia and hypertriglyceridemia. However, the high fructose diet caused steatosis in the male and female rats and caused hepatic lipid accumulation. The zingerone prevented the high-fructose diet induced steatosis in both male and female rats but failed to protect the rats against the dietary fructose induced hepatic lipid accretion. In female rats zingerone caused visceral adiposity. The administration of fenofibrate protected the rats against the dietary fructose induced steatosis and hepatic lipid accumulation. However in male rats fenofibrate caused liver hepatomegaly, hypoglycaemia, decreased insulin homeostasis and lowered insulin sensitivity. In female rats, fenofibrate caused hypertriglyceridemia.

Body weight can be used as an index for general obesity or overall health status of an animal (Nuttall, 2015). On the other hand, eviscerated carcass weight is considered a surrogate for assessing lean body weight (Miller et al., 2011). Whereas an increase in visceral fat pad weight indicates central obesity and is associated with the onset of metabolic derangements (Item and Konrad, 2012). The results of my study show that feeding a high-fructose diet for 12-weeks did not affect the body weight gain, empty carcass weight and visceral adiposity of growing male and female rats. Lembede et al. (2018) reported that feeding male and female rats a high-fructose diet for 12-weeks caused visceral adiposity but did not affect the rats' body weight gain and empty carcass weight. The young age of the rats that were used in my study could be the reason why the high-fructose diet did not result in central obesity. It has been shown that at a younger age, rats are more resistant to developing high-fructose diet-induced visceral adiposity (Ibrahim et al., 2017). The results of this study also show that in high-fructose diet-fed female rats the administration of zingerone resulted in increased visceral adiposity. However, zingerone had no effect on the body weight gain and empty carcass weight of male and female rats. Zingerone is known to exert anti-obesogenic effects by upregulating hormone-sensitive lipase-induced lipolysis in fructose-fed rats (Narayanan et al., 2016). My results show that in female rats zingerone had obesogenic effects. This finding goes against current literature. I speculate that due to differences in sex hormones between

male and female rats, in females zingerone's stimulatory effects on hormone-sensitive lipase are suppressed resulting in the accumulation of lipids in adipose tissue. This therefore needs further investigation, especially at a molecular level. Moreover, my findings suggest that zingerone can be administered to growing male and female rats, without adversely affecting body weight gain and empty carcass weight. My study also found that the administration of fenofibrate, as an intervention, resulted in reduced body weight gain and empty carcass weight in high-fructose diet-fed male rats, but not in the female counterparts. Similarly, Lembede et al. (2019) reported that in high-fructose diet-fed male rats, fenofibrate reduced body weight gain and empty carcass weight but did not affect visceral adiposity. Dotson et al. (2016) reported that female mice have lower PPAR-α expression in their tissues compared to their male counterparts. Thus, it is possible that fenofibrate did not reduce body weight gain and empty carcass weight in female rats due to the lower expression of PPAR-α in their tissues. Importantly, my findings suggest that fenofibrate should be utilized warily, as it might compromise body weight gain and empty carcass weight.

Body weight measurements can be used to evaluate the growth of an animal (Diina, 2019). However, body weight is a non-precise tool for assessing growth since it can be transiently influenced by fasting state, hydration state, gut fill as well as organ mass and does not take into account the distribution and proportions of lean and fat weight (Reilly et al., 2000; Borga et al., 2018; Shuster et al., 2012). As a result, long bone indices are considered a more accurate proxy for assessing growth in animals (Ruff, 2003; Eshet et al., 2004). Prior to epiphyseal fusion, long bone growth is positively correlated with the growth hormone (Dennison et al., 2003; Eshet et al., 2004). In my study, tibia weight, length and weight to length ratio were not affected by the high-fructose diet, zingerone and fenofibrate. My findings are similar to those by Nyakudya et al. (2019), who reported that high fructose feeding did not compromise long bone indices in rats. Additionally, my findings suggest that zingerone did not adversely affect the long bone indices in high-fructose diet-fed male and female rats.

Fructose metabolism results in increased triglycerides *de novo* synthesis which results in elevated lipid accretion in the liver and consequently the development of fatty liver disease (Park et al., 2013). In my study, feeding a 20% fructose solution to rats for 12 weeks caused increased hepatic lipid accretion. The increased liver lipid accumulation was accompanied by macro-vesicular hepatic steatosis in both male and female rats. My findings therefore suggests that fructose feeding to male and female rats enhanced hepatic *de novo* lipogenesis,

and this led to elevated hepatic lipid build-up and subsequently the development of steatosis. These findings from my study are consistent with those by Gumede et al. (2020) who reported that feeding a high-fructose diet to young rats for 12 weeks caused the development of NAFLD. Although orally administered zingerone did not prevent increased liver lipid accretion in high-fructose diet-fed rats, it did prevent the development of steatosis in the rats. Naidu et al. (2018) reported that the oral administration of 100 mg/kg zingerone prevented steatosis in rats fed 40% fructose using rodent chow and drinking water (25% fructose). Naidu et al. (2018) further reported that zingerone exerted it protective effect by increasing its scavenging activity in the liver. Therefore, it is possible that in my study the zingerone prevented the development of steatosis by upregulating it antioxidant activity. Moreover, the manifestation of hepatic steatosis is regulated by a lipid partitioning enzyme, stearoyl-CoA desaturase-1 (SCD-1); (Li et al., 2009). Therefore, I hypothesise that zingerone prevented the formation of steatosis in high-fructose diet-fed rats by modulating the mechanism of action of SCD-1. On the other hand, fenofibrate prevented the high-fructose diet-induced increase hepatic lipid accretion in male rats, but only attenuated the increased hepatic lipid accretion in female rats. Additionally, like zingerone, fenofibrate prevented the formation of hepatic steatosis in the high-fructose diet-fed male and female rats. Importantly, fenofibrate is known to prevent diet-induced fatty-liver diseases through the activation of PPAR-α and lipoprotein lipase (Pan et al., 2012), evidently fenofibrate prevented formation of hepatic steatosis in my study.

In hepatocytes aspartate aminotransferase is a transaminase enzyme that catalyses the transfer of amino groups during gluconeogenesis and amino acid metabolism (Lee et al., 2008). Thus, an increase in plasma AST activity signifies hepatocellular damage (Lee et al., 2008; Beek et al., 2013) and injury (Pratt and Kaplan, 2000). My study findings show similarities in the plasma AST activity of the rats across treatment regimens. It must be noted that AST is produced and regulated by the liver, thus higher than normal plasma AST activity signifies hepatocellular damage/injury. Fatty-liver disease is correlated with hepatocellular damage and increased plasma AST activity (Gowda et al., 2009; Hyder et al., 2013). However, plasma AST activity typically increases in the advanced stages of the fatty-liver disease which are associated with hepatocellular inflammation and damage as well as reduced liver function (Thapa and Walia, 2007; Preiss and Sattar, 2008). In my study, the high-fructose diet, zingerone and fenofibrate had no effect on the plasma AST activities of the rats. My findings suggests that the high-fructose diet, zingerone and fenofibrate did not cause

hepatocellular damage nor compromise liver function in the rats. Previous studies have reported that high-fructose diet-induced fatty-liver disease can result in hepatocellular damage, an increase in plasma AST activity (Poudyal et al., 2010; Wang et al., 2015). However, in these studies, the high-fructose diet resulted in more severe fatty-liver diseases (Poudyal et al., 2010; Wang et al., 2015). In its early stages, the fatty-liver disease is typified by increased liver lipid accumulation and steatosis, without hepatocyte inflammation and or damage (Benedict and Zhang, 2017). Indeed, in my study, the high-fructose diet caused fatty-liver disease, however, inflammation and hepatocyte injury were not observed in the liver sections of male and female rats. I, therefore, speculate that in my study, the high-fructose diet caused mild or early-stage fatty-liver disease without compromising liver function. Notably, my findings also suggest that zingerone and fenofibrate can be used as an intervention for diet-induced steatosis without compromising liver health. The liver plays a vital role in the metabolism and detoxification of orally administered substances (Sinturel et al., 2017) and thus, dietary interventions can have an impact on liver weight. Despite the high-fructose diet causing increased liver lipid accretion in the rats, it had no effect on their liver weights. Similarly, zingerone did not affect the liver weights of male and female rats. On the contrary, fenofibrate increased the liver weights of male and female rats. Lembede et al. (2019) also reported that the administration of 100 mg/kg fenofibrate body weight per day, to growing rats caused hepatomegaly. It was reported that fenofibrate increases liver weight through its proliferative effects on liver tissue (Huang et al., 2013). Thus, based on my findings, it is possible that fenofibrate administration caused hepatomegaly in rats through hepatic hepatocyte proliferation since AST activity was not affected. Crucially, my findings suggest that fenofibrate should be used with care as it could possibly cause hepatomegaly in male rats.

An increase in hepatic lipid accretion results in increased hepatic lipid efflux into circulation (VerHague et al., 2013) thus fatty-liver disease is correlated with an increase in blood tryglycerides and cholesterol (Pacifico et al., 2014; Sun et al., 2015). Despite the high-fructose diet causing increased liver lipid accumulation in my study, it did not cause hypertriglyceridemia and hypercholesterolemia. The degree of lipid saturation in visceral adipose stores determines the development of hypertriglyceridemia and hypercholesterolemia in high-fructose diet-induced fatty liver disease (Ter Horst and Serlie, 2017). A higher saturation with lipids in visceral adipose stores increases the likelihood that hypertriglyceridemia and hypercholesterolemia will develop in high-fructose diet-induced

fatty-liver disease rats (Khitan and Kim, 2013; Ter Horst and Serlie, 2017). Therefore, in my study, the lack of visceral obesity may explain why the high-fructose diet did not cause hypertriglyceridemia and hypercholesterolemia in the rats. Zingerone had no effect on the plasma triglyceride and cholesterol concentrations of high-fructose diet-fed rats. On the contrary, fenofibrate increased the plasma triglyceride concentration of fructose-fed female rats only but did not affect their plasma cholesterol concentration. Fenofibrate exerts its anti-obesogenic effects by increasing lipolysis in tissues, which can result in an influx of triglycerides into circulation (Ferreira et al., 2006). Thus, I speculate that in my study fenofibrate increased plasma triglycerides concentration by upregulating lipolysis in a sex-dependant manner. Nonetheless, further interrogations are required to fully comprehend the mechanisms by which fenofibrate increased plasma triglycerides concentration. The consumption of fructose rich diets is reported to cause glucose homeostasis disturbances, insulin resistance and hypoadiponectinemia (Putakala et al., 2017). However, these metabolic derangements typically manifest if the fructose causes visceral obesity and dyslipidaemia (Ferder et al., 2010; Rodríguez et al., 2016). Visceral obesity is positively correlated with hypoadiponectinemia which causes insulin resistance (Di Chiara et al., 2012). Adiponectin is an anti-inflammatory adipokine synthesized by adipocytes and it stimulates fatty-acid oxidation and improves insulin sensitivity (Silva et al., 2014). My findings show that the high-fructose diet did not affect plasma adiponectin and insulin concentrations and insulin resistance. A possible explanation for this finding could be that the fructose solution did not cause visceral obesity in the rats and hence their plasma adiponectin and insulin concentrations and insulin resistance were not affected. The oral glucose tolerance test and its area under the curve are used to evaluate glycaemic homeostasis proficiency (Pruessner et al., 2003; Tschritter et al., 2003). The findings from the oral glucose tolerance test and its area under the curve suggest that the high-fructose diet did not affect glycaemic homeostasis. I speculate that since the high-fructose diet did not affect plasma insulin concentration and insulin sensitivity, glucose homeostasis was maintained in the rats. During the oral glucose tolerance test the high-fructose diet-fed rats that received zingerone, as an intervention, returned to basal sooner than those of rats that received other treatments. However, the zingerone had no effect on the area under the curve of the oral glucose tolerance test, plasma insulin and adiponectin concentrations and insulin resistance in the high-fructose diet-fed male and female rats. My findings suggest that zingerone may exert propitious effects on glycaemic control in a sex dependent manner. Nonetheless, since zingerone did not affect the area under the curve of the oral glucose tolerance test further interrogations are necessary to

fully establish these potential beneficial effects on glycaemic control by zingerone. Fenofibrate increased glucose and insulin concentrations and diminished glycaemic control and insulin sensitivity in fructose-fed male rats in my study. Oosterveer et al. (2009) reported that fenofibrate decreases hepatic glucose uptake and increased hepatic gluconeogenesis from glycerol. Thus, I speculate that in my study, fenofibrate increased blood glucose concentration and dysregulated glycaemic control in the rats by stimulating gluconeogenesis, causing insulin resistance which consequently resulted in increased plasma insulin concentration. However further investigations are required to establish the mechanism of action by which fenofibrate increases glucose and insulin related disturbances in the rats.

# Chapter Six: Conclusion, Limitations and Recommendations

In this study I evaluated the protective effect of zingerone against growth performance, glucose tolerance and HOMA-IR index, visceral adiposity, plasma cholesterol, triglycerides, insulin, adiponectin concentration and plasma AST activity and liver weight, and NAFLD in fructose-fed young rats.

The findings from my study showed that the consumption of a high-fructose diet for 12 weeks resulted in fatty-liver disease typified by increased hepatic lipid accretion and caused hepatic steatosis and in growing rats. Nonetheless, the fructose did not affect growth performance and did not compromise glycaemic control or cause dyslipidaemia, visceral obesity and or insulin resistance. Taken together these findings suggest that fructose caused fatty-liver disease without causing MetS. Moreover, these findings support previous findings which showed that fatty-liver disease and MetS can occur independently (Meng et al., 2018; Xie et al., 2013). Zingerone did not prevent the fructose-induced increase in liver lipid content. Nonetheless, zingerone prevented the formation of steatosis in the fructose-fed rats. Overall, these findings suggest that zingerone may have modulatory effects on liver lipid partitioning enzymes which prevented the formation of steatosis despite the increase in liver lipid accretion. Zingerone potentially utilized it anti-steatotic in preventing the formation of steatosis in the liver. Nonetheless, further investigations are needed to establish how zingerone prevented the formation of steatosis. Surprisingly, when female rats that received the high-fructose diet with zingerone, as an intervention, had an increase in visceral fat pad weight. Currently, this finding is challenging to explain, however, I hypothesise that zingerone may potentiate the obesogenic effects of fructose in female rats. This observation needs further investigation to establish the underlying mechanisms involved in this outcome. Importantly, zingerone had no effect on growth performance, glycaemic control, plasma cholesterol, triglycerides, insulin and adiponectin concentrations, AST activity and HOMA-IR. Thus, zingerone could be used for its beneficial medicinal effects without adversely affecting growth, circulating metabolites, metabolism-regulating hormones and liver health. In male rats, fenofibrate caused a decreased in the terminal and empty carcass weights, hepatomegaly, insulin resistance and glycaemic control dysregulation. Additionally, in the female rats, fenofibrate caused hypertriglyceridemia and hepatomegaly. The adverse effects elicited by fenofibrate in my study have been reported previously (Yoon et al., 2002; Lembede, 2014). Thus, my findings suggest that caution should be exercised when using fenofibrate.

In my study, a single dose of zingerone was investigated in high-fructose diet-fed rats. Thus, my study did not interrogate the potential dose-effects of zingerone. I recommend that future studies interrogating the beneficial medicinal effects of phytochemicals should include the low and high dose in their study design. Moreover, this study did not conduct any molecular investigations which could have possibly provided insights into the mechanisms involved in some of the findings from my study. Therefore, future investigations on the potential anti-obesogenic and antidiabetic effects of zingerone should include molecular interrogations. My study did not investigate the effects of the treatments on liver lipid partitioning enzymes, thus the mechanism by which zingerone prevented steatosis, despite the increase in liver lipid accretion, are not fully understood. Further investigations are necessary to understand the mechanisms of action of zingerone in the liver.

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# Appendices

## Appendix I: Animal Ethics Clearance

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

November 21, 2018

**Certificate reference**: 2017 010 71/B

**Applicant : Busisani W Lembede**
**Department:** Physiology
**Tel:** +27 (0)11 717 2154 ; **Email:** Busisani.Lembede@wits.ac.za

**RE: Clarification/recommendations to M&E request**

**Title**: The potential of orally administered Zingerone to protect and to programme for protection against high-fructose high-cholesterol diet-induced metabolic derangements in Sprague-Dawley rats.

**Clarification**:
The study is to be shortened – the end point is now going to be adolescence rather than late adulthood. The request is to continue with fructose solution straight after weaning rather than give a break before recommencing with fructose. Therefore the continual exposure to fructose without a break means that the study does not have to go on for so long.

Study numbers – 300 rats allocated & 134 used leaving 166 allocated but not used.
Expt 1 used 100 rats and the study has been completed
Expt 2 allocated 200 rats - 34 had been allocated to phase 2 at the time of application. The study is still in the early phase of gavaging as originally approved. M&E reduces study number to 80.

DMSO no longer required - warming in water to 41C solubilises the zingerone and hence DMSO is no longer required as a solvent.

- **APPROVED** November 21, 2018

GP Candy

Geoffrey Candy
Chair : Animal Ethics Research Committee, University of the Witwatersrand

*Geoffrey P Candy PhD*
*Professor, Department of Surgery, Faculty of Health Sciences,* University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*

## Appendix II: Elisa Protocols

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7th Edition, revised in April, 2017

**(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)**

**Rat ADP/Acrp30(Adiponectin) ELISA Kit**

Synonyms: Acrp30, GBP28, ACDC, APM1, ADPN, AdipoQ, ADIPQTL1

Catalog No : E-EL-R0329
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
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**SUMMARY**

1. Add 100 μL standard or sample to each well. Incubate for 90 min at 37°C.
2. Remove the liquid. Add 100 μL Biotinylated Detection Ab. Incubate for 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 μL HRP Conjugate. Incubate for 30 min at 37°C.
5. Aspirate and wash 5 times.
6. Add 90 μL Substrate Reagent. Incubate for 15 min at 37°C.
7. Add 50 μL Stop Solution. Read at 450 nm immediately.
8. Calculation of results.

## Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operational skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions!
4. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from the above reasons, too.
7. Valid period: 6 months.

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# Rat INS (Insulin) ELISA Kit

Catalog No: E-EL-R2466

96T

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## Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** It's recommended that all samples and standards be assayed in duplicate.

1. **Add Sample:** Add 100μL of Standard, Blank, or Sample per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃.
2. **Biotinylated Detection Ab:** Remove the liquid of each well, don't wash. Immediately add 100μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. **Wash:** Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350μL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
4. **HRP Conjugate:** Add 100μL of HRP Conjugateworking solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C.
5. **Wash:** Repeat the wash process for five times as conducted in step 3.
6. **Substrate:** Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.
7. **Stop**: Add 50μLof Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8. **OD Measurement**: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.
9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

## Important Note:

1. **ELISA Plate**: The just opened ELISA Plate may appear water-like substance, which is normal and will not have any impact on the experimental results.
2. **Add Sample**: The interval of sample adding between the first well and the last well should not be too long, otherwise will cause different pre-incubation time, which will significantly affect the experiment's accuracy and repeatability. For each step in the procedure, total dispensing time for

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# Total Cholesterol Assay Kit (Single reagent, COD-PAP method)

**Catalog No:** E-BC-K109
**Method:** Colorimetric method
**Specification:** 100 tubes/96 samples

This manual must be read attentively and completely before using this product.

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## Application

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

## Detection principle

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

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

$$4 - AAP + H2O2 + 3,5 - DHBS \xrightarrow{POD} \text{Red quinone} + H2O$$

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

## Kit components

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

## Sample Preparation

1. **Serum (Plasma):** Detect the sample directly. If the concentration is beyond the linear range, then dilute the sample with saline before detection.
2. **Culture fluid sample:** Collect the culture medium, centrifuge at 1000 rpm for 10 min, and take the supernatant for detection.
   **[Note]: It is generally recommended that the cell density should be more than $1\times10^6$ /mL.**
3. **Tissue sample:** Accurately weigh the tissue weight, add 9 times the volume of homogenate media according to the ratio of Weight (g): Volume (mL) =1:9. Mechanical homogenate the sample in ice water bath. Centrifuge at 2500 rpm for 10 min, then take the supernatant for detection.
   **[Note]: (1) If the tissue sample is not a high-fat sample, the homogenate media should be phosphate buffer (0.1 mol/L, pH 7.4) or normal saline.**

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# Triglyceride Assay Kit (Single reagent, GPO-PAP method)

**Catalog No:** E-BC-K261
**Method:** Colorimetric method
**Specification:** 100 tubes/96 samples

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## Product description

This kit applies the GPO-PAP method and it can be used for *in vitro* determination of triglyceride (TG) content. It can be operated with all kinds of spectrophotometer.

## Detection principle

$$\text{Glycerin trilaurate} + \text{H20} \xrightarrow{\text{Lipase}} \text{Glycerinum} + \text{Fatty acid}$$

$$\text{Glycerinum} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol} - 3 - \text{phosphoric acid} + \text{ADP}$$

$$\text{Glycerol} - 3 - \text{phosphoric acid} + \text{O2} \xrightarrow{\text{Glycerol-3-Phosphate Oxidase}} \text{Hydroxyacetone phosphate} + \text{H2O2}$$

$$\text{Parachlorphenol} + \text{H2O2} + 4 - \text{AAP} \xrightarrow{\text{Peroxidase}} \text{Red quinone}$$

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

## Kit composition

| Composition | Size | Component | Concentration | Storage |
|---|---|---|---|---|
| Working Solution (Enzyme) | 100 mL × 1 vial | Tris-HCL Buffer | 100 mmol/L | 2-8℃ (shading light) |
| | | Lipase | ≥ 3000 U/L | |
| | | ATP | 0.5 mmol/L | |
| | | Glycerol Kinase | ≥ 1000 U/L | |
| | | Glycerol-3-Phosphate Oxidase | ≥ 5000 U/L | |
| | | Peroxidase | ≥ 1000U/L | |
| | | 4-Aminoantipyrine | 1.4 mmol/L | |
| | | Parachlorphenol | 3 mmol/L | |
| Standard | 0.1 mL × 1 vial | Glycerinum | 2.26 mmol/L | |

## Experimental instruments

Test tube, Micropipettor, Centrifuge, 37℃ water bath, Spectrophotometer (510 nm)

## Sample preparation

1. **Serum (Plasma):** Detect the sample directly. If the concentration is beyond the linear range, then dilute the sample with normal saline before detection.
2. **Culture fluid sample:** Draw the culture medium, centrifuge at 1000 rpm for 10 min, and take the supernatant for detection.
   **[Note]: It is generally recommended that the cell density should be more than $1\times10^6$/mL.**
3. **Tissue sample:** Accurately weigh the tissue weight, add 9 times the volume of **homogenate media** according to the ratio of Weight (g): Volume (mL) =1:9. Mechanical homogenate the sample in ice water bath. Centrifuge at 2500 rpm for 10 min, then take the supernatant for detection.
   **[Note]: (1) If the tissue sample is not a high-fat sample, the homogenate media should be phosphate buffer (0.1 mol/L, pH 7.4) or normal saline.**
   **(2) If the tissue sample is high-fat sample or partly high lipid sample, the homogenate media should be absolute alcohol.**
4. **Cell sample:**
   **Cell collection:** Take the prepared cell suspension and centrifuge at 1000 rpm for 10 min. Discard the supernatant and keep the cell sediment. Wash the sediment with iso-osmia buffer (0.1 mol/L, pH7~7.4 phosphate buffer was recommended) 1~2 times, centrifuge at 1000rpm for 10min. Discard the supernatant and keep the cell sediment.
   **Cell disruption:** Add 0.2~0.3 mol/L of homogenate media (0.1 mol/L, pH7~7.4 phosphate buffer or normal saline was recommended). Sonicate in ice water bath (power: 300 W, 3~5 second/time, interval for 30 sec, repeat for 3~5 times) or grind with hand-operated. The prepared homogenate kept for detection without centrifugation. The cell can also be lysed with the cell lysate buffer (Triton X-100, 1~2%, lysate for 30~40 min), then take the prepared lysate for detection directly without centrifugation.
   **[Note]: It is generally recommended that the cell density should be more than$1\times10^6$/ml. The disrupted cell can be observed with microscope that whether the cell is broken completely.**

## Operation steps

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

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