QUERCETIN AS PROPHYLAXIS AGAINST HIGH FRUCTOSE DIET-INDUCED NON-ALCOHOLIC FATTY LIVER AND PANCREATIC DISEASE IN FEMALE SPRAGUE DAWLEY RATS

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DECLARATION

I, Abubakar Namadina Muhammad, declare that this dissertation is my own, except where others have assisted me as acknowledged. This dissertation is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other University. All the experimental procedures used in this dissertation were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2017/02/07/B).

Abubakar Namadina Muhammad

Signed on the18th of June, 2021

DEDICATION

I have devoted this research work with utmost respect, love and affection to my beloved parents (Alhaji Namadina Ahmad Sokoto and Hajiya Halimatu Ismail), as well as my darling wife (Mufida Yakubu Umar Mani), my lovely children (Abdullah and Abdurrahman) and my brothers and sisters.

ABSTRACT

The rising worldwide ingestion of fructose enriched diets has coincided with the global epidemics of diabetes mellitus, obesity and ectopic lipid deposition in other organs such as the liver and pancreas. The fructose diet induced lipid deposition in the liver is termed non-alcoholic fatty liver disease (NAFLD) and often coexists with the lipid deposition in the pancreas which is known as non-alcoholic fatty pancreas disease (NAFPD). The prevalence of NAFLD and NAFPD is estimated at 25% and 16% in the general population respectively, and is rising steadily. Posing huge health and economic challenge, and yet no standard approved treatment guidelines. The few, synthetic drugs being used to treat these diseases are associated with significant adverse side effects like constipation, hepatitis, and pancreatitis. Hence, the current study explored the potential of the phytochemical quercetin in preventing the occurrence of NAFLD and NAFPD, as well as the metabolic dysfunction associated with a high fructose feeding in growing female Sprague Dawley rats. Thirty seven, 21 day old, female Sprague Dawley rats were randomised into one of four experimental groups and fed for with their respective diets and treatments. Control group (C) received SRC (Standard rat chow), tap water, and plain gelatine cubes daily; quercetin group (Q) received SRC, tap water, and quercetin cubes (100 mg/kg body weight) daily; fructose group (F) received SRC, and 20% fructose solution (FS) to drink *ad libitum,* and plain gelatine cubes daily; fructose plus quercetin group (F + Q) received SRC, 20% FS *ad libitum* and quercetin cubes (100 mg/kg body weight) daily. At the end of 12 weeks feeding and treatments, the animals were terminated; blood and tissues samples were collected.

The growth performance was unaffected ($P > 0.05$), however, fructose consumption significantly ($P < 0.05$) reduced food consumption and increased overall total caloric intake, visceral fat mass, liver mass, hepatosomatic index (HSI), hepatic lipid yield, and induced NAFLD. However, the fructose feeding did not induce NAFPD or insulin resistance and except for the reduced high density lipoprotein cholesterol (HDL-C) level, all other circulating metabolic substrates were unaffected ($P > 0.05$). Quercetin did not improve the visceral fat mass, liver mass and HSI, but improved the HDL-C level, hepatic steatosis, inflammation and fibrosis significantly ($P < 0.05$). There was a trend towards the reduction of hepatic lipid yield below the 10% mean value cut off mark applicable for the diagnosis of NAFLD. Red blood cell indices, markers of liver, and renal functions were unaffected by either the fructose solution or quercetin

treatment, and no adverse effects were observed. Therefore, the use of quercetin as a preventative intervention against high fructose induced NAFLD should be further examined as a natural alternative in curtailing the rising prevalence of dietary fructose induced NAFLD globally.

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LIST OF ABBREVIATIONS

ALT: ......Alanine aminotransferase
AMP: .....Adenosine monophosphate
AMPK:.....Adenosine monophosphate-activated protein kinase
AST: .....Aspartate aminotransferase
ATP: .....Adenosine triphosphate
BMI: ....Body mass index
BUN: ..... Blood urea nitrogen
C: .....Control group
ChREBP:..... Carbohydrate responsive element-binding protein
CT:..... .....Computed tomography
DAG: .....Diacylglycerol
ELISA: ..... Enzyme linked immuno sorbent assay
EUS: ..... Endoscopic ultrasound
F + Q:..... Fructose plus quercetin group
F: ..... Fructose group
FBG: .....Fasting blood glucose
FFAs:.....Free fatty acids
FPG: .....Fasting plasma glucose
FPI: ..... Fasting Plasma Insulin
FS:..... Fructose solution
GFR:..... Glomerular filtration rate
Hb: ..... Haemoglobin
HCt: .....Haematocrit
HDL-C:.....High density lipoprotein cholesterol
HE:..... Haematoxylin and eosin
HFCS:.....High fructose corn syrup
HOMA-IR: ..... Homeostatic model of assessment of insulin resistance
IL: .....Interleukin
KHK:.....Fructokinase
LDL-C:..... Low density lipoprotein cholesterol
MCHC:..... Mean corpuscular haemoglobin concentration
MRI: .....Magnetic resonance imaging
MT: ..... Masson’s trichrome

NAFLD: ..... Non-alcoholic fatty liver disease
NAFPD: ..... Non-alcoholic fatty pancreas disease
NAS: ..... NAFLD activity score
NASH: ..... Non-alcoholic steatohepatitis
NFS: ..... NAFLD fibrosis score
NFκB/IκBα: ..... Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
OD: ..... Optical densities
PCV: ..... Packed cell volume
PFK I: ..... Phosphofructokinase I
PPARγ: ..... Peroxisome proliferator-activated receptor gamma
Q: ..... Quercetin group
ROS: ..... Reactive oxygen species
SIRT1: ..... Silent information regulator 1
SRC: ..... Standard rat chow
SREBP-1c: ..... Sterol regulatory element-binding protein-1c
TAG: ..... Triacylglycerol
TC: ..... Total cholesterol
TNF: ..... Tumor necrosis factor
TW: ..... Tap water
USG: ..... Ultrasonography
VLDL: ..... Very low density lipoprotein

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The increased consumption of diets high in refined fructose such as junk food and high fructose containing drinks has been closely linked with the current global epidemics of obesity, diabetes mellitus, and dyslipidaemia in adults, adolescents, and children (Bentley et al., 2020). Poor lifestyle choices including sedentary habits coupled with the high dietary fructose intake predispose individuals to the development of not only metabolic syndrome and its components. As well as the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic fatty pancreas disease (NAFPD) across all genders, race and age groups (Wong and Chan, 2020).

Currently, the global prevalence of NAFLD and NAFPD is about 25% and 16%, respectively (Wong and Chan, 2020). However, the prevalence even higher in individuals with other co-morbidities such as metabolic syndrome and diabetes mellitus (Yu et al., 2019b). Currently, there are no approved standard treatment protocols for treating NAFLD or NAFPD. Although some synthetic drugs such as metformin, pioglitazone and statins are used in treating these diseases. These drugs are expensive and often come with concomitant undesirable negative side effects such as hepatitis, pancreatitis, erectile dysfunction and peripheral neuropathy (Iogna Prat and Tsochatzis, 2018). Hence, there is a need for treatments and prophylactic agents that have high efficacy, are relatively less expensive, have fewer adverse side effects, and are naturally (plants) sourced (Yang et al., 2019).

Plant derived phytochemicals have been in use as prophylactic and therapeutic agents in many communities globally for decades in addressing their health needs (Yao et al., 2016). There are a great number of studies evaluating the use of phytochemicals in the treatment of different ailments, due to their natural origin, holistic nature of therapy, and perceived reduced side effects (Yang et al., 2019). However, currently the studies on the use of phytochemicals in the management of NAFLD and NAFPD are insufficient, and most of these studies which make use of animal models, are biased to the use of adult male rats, neglecting the sexual dimorphism of these metabolic disorders. Consequently, the current study investigated the prophylactic ability of the phytochemical, quercetin, in preventing the occurrence of metabolic dysfunction (NAFLD and NAFPD) caused by high fructose diet ingestion in growing female, Sprague Dawley rats.

The dissertation consists of five chapters namely; chapter one which consists of an introduction to NAFLD, NAFPD, and quercetin. The typical events leading to metabolic dysfunction (NAFLD and NAFPD) due to high fructose intake is also discussed in this chapter, as well as the rationale and aims and hypotheses of the current study. Chapter two details the materials and methodology employed to actualize the set objectives. Chapters three and four consist of the results obtained and a discussion of the findings, respectively. Chapter five comprises of conclusions, limitations, and recommendations arising from the study outcomes and chapter six consists of the list of references. Supplementary information is provided in the appendices.

1.2. Dietary sources of fructose and consumption patterns

Excess dietary sugar intake such as fructose has been linked with deleterious metabolic effects (Elshazly et al., 2020). Fructose, a simple sugar (monosaccharide) is an isomer of glucose, naturally present in many plants, including sugar cane, sugar beets, and corn (Yilmaz, 2012). This raw fructose from plant products can undergo refining processes to form a fine, clear, crystalline, and very sweet powder that forms part of sucrose (table sugar). That can be further converted into more concentrated forms such as in high fructose corn syrups (HFCS) and fruit juice concentrates (Chen et al., 2020a, Macdonald, 2020). Fructose, particularly in its refined form, has been used as a substitute for sucrose as an added sugar in processed foods and beverages (Macdonald, 2020). Because it is very cheap, sweet, and has a high solubility (Amaral-Fonseca et al., 2020).

The global per capita fructose consumption was very low before the early 1970s when refined fructose (in the form of high fructose corn syrup) was initially utilised in the food and drink processing industries. Since then the global, per capita fructose consumption significantly increased to over 60 pounds (27.3kg) by the year 2000. However, data about the fructose consumption patterns of individual countries is insufficient (Bentley et al., 2020). In the USA, a study on national dietary data reported mean fructose intake to be around 37 g/day (8% of total Americans' caloric consumption) between the years 1977 to1978. Reports that are more recent estimated the mean consumption of fructose between 1978 and 2004 to be 54.7 g/day (10% of total Americans' daily caloric ingestion). The fructose intake was highest among teenagers (12 to18 years) at 72.8 g/day (12.1% of total daily energy

demand) (Miriam et al., 2008, Alwahsh and Gebhardt, 2017). However, survey reports from 2005 indicated that consumption of fructose in the form of HFCS is decreasing in the USA, but fructose in the form of table sugar (fructose in combination with glucose) is still being consumed at a high level (Rippe and Angelopoulos, 2013, Rippe, 2014). Data obtained from different regions of the world by the Food and Agriculture Organization (FAO) of the United Nations, showed an increase in the per capita sugar consumption from 1963 to 2003 and future projections (2025 and 2050) among different regions of the world, if attitudes towards sugar consumption remain unchanged (Table 1.1) (Kearney, 2010, Rippe, 2014).

Table 1.1 Refined sugar consumption trends and projections in different regions of the world (g/capita/day) (Kearney, 2010, Rippe, 2014)

Regions/ Years	**1963**	**1973**	**1983**	**1993**	**2003**	**2025**	**2050**
Africa	24.7	30.1	35.6	32.9	38.4	42.2	49.7
Sub-Saharan Africa	13.7	19.2	21.9	19.2	24.7	30.6	36.1
Asia	11.0	16.4	21.9	30.1	35.6	49.1	64.8
Europe	82.2	98.6	93.2	87.7	93.2	96.4	99.1
Eastern Europe	68.5	90.4	98.6	79.5	84.9	95.2	100.7
China	2.7	5.5	13.7	11.0	16.4	26.1	34.4
India	13.7	13.7	21.9	35.6	43.8	13.5	17.0

Countries in Southern Africa are not excluded from the current global increase in fructose consumption. Fructose per capita consumption has been estimated to have been in the range of 80 to 108 g/capita/day between 1992 to 2007 with Botswana, South Africa, and Swaziland having the highest sugar/sweetener consumption in the region (Nnyepi et al., 2015). In South Africa, similar to most countries globally, the fructose intake has exceeded the World Health Organisation recommended daily sugar intake of < 10% of the total daily energy requirement (Nnyepi et al., 2015).

Reports on children (aged 12 to 24 months) living in urban South Africa showed that daily fructose intake in the form of sweetened carbonated soft drinks to be between 54.3 to 86.2 g/child/day (Nnyepi et al., 2015). Another study reported an increase in sugar (fructose and glucose) consumption in children and adolescents to about 50 g/day and 100 g/day, respectively (Nnyepi et al., 2015, Maarman et al., 2016). Since these global trends of increased fructose consumption coincides with the current global epidemics in obesity, diabetes mellitus, insulin resistance, dyslipidaemia, NAFPD, and NAFLD. Studies in humans and animals have been focussed on the effects of increased fructose consumption as the prime suspect (Bentley et al., 2020, Elshazly et al., 2020, Gumede et al., 2020b). Therefore, since the increased consumption of fructose has been linked with dire metabolic consequences that arise from fructose breakdown, specifically hepatic fructolysis, the next section will focus on hepatic fructose metabolism.

1.3. Fructose Metabolism and its Contribution to Metabolic Disorders

1.3.1. Hepatic metabolism of fructose

The liver cells are the major site of fructolysis; key metabolic processes include fructolysis and lipid metabolism. Fructose metabolism in the liver occurs after ingestion and absorption of fructose from the intestinal lumen to the hepatocytes via the portal circulation, by the fructose specific GLUT 5 and GLUT 2 transporters (Soleimani, 2011, Yilmaz, 2012). The unrestricted and insulin independent fructokinase enzyme present in the hepatocyte rapidly phosphorylate fructose to fructose1phosphate (Elliott et al., 2002, Tappy and Le, 2010). Fructose1phosphate is then converted to glyceraldehyde and dihydroxyacetone phosphate in a reaction catalyzed by aldolase B. These are further, converted to either glycerol3phosphate or glyceraldehyde 3phosphate (G3P), which subsequently enters the glycolytic metabolite pools to be used as the backbone for fatty acids, phospholipids and triacylglycerol (TAG) formation (Elliott et al., 2002, Tappy and Le, 2010).
Different from glucose metabolism, fructolysis is unrestricted by the rate limiting enzyme phosphofructokinase I (PFK I) (Koo et al., 2008), as a result, liver fructolysis produces a large number of precursors for glycolytic pathways, that end as lactate, which in turn serves as raw material for acetyl-CoA formation which is subsequently used in the production of TAGs and very low density lipoprotein (VLDL) (Hannou et al., 2018, Tamer et al., 2020). Therefore, since fructolysis is uncontrolled, excessive

dietary fructose intake will lead to the continuous production of lipid by-products that will subsequently lead to the development of various metabolic dysfunctions.

1.3.2. The role of fructose in causing metabolic dysfunctions

The unique property of fructose as an obesogenic agent (in that it negatively impacts lipid metabolism) (Buzzetti et al., 2016) is noted above. Excessive dietary fructose consumption will result in increased lactate formation and enhanced hepatic *de novo* lipogenesis with resultant dyslipidaemia, increased fat deposition, and hepatic lipid deposition (Elliott et al., 2002, Johnson et al., 2020). Fructose metabolism is also known to facilitate esterification of preformed free fatty acids (FFAs) from adipose tissues or diet. As well as suppression of hepatic fatty acid oxidation and VLDL secretion, leading to increased hepatic lipid accumulation and tissue specific insulin resistance, especially in the liver and adipose tissues (Softic et al., 2020). Fructolysis also enhances the production of triosephosphates which are channelled to central carbon metabolic pathways, such as glycolysis and gluconeogenesis, leading to hyperglycaemia and increased glycogen production (Johnson et al., 2020).
The first step of fructolysis (i.e. the formation of fructose1phosphate from fructose) consumes a lot of energy (adenosine triphosphate (ATP)) resulting in hepatic energy depletion and increased adenosine monophosphate (AMP) formation. Which is a precursor of uric acid production, leading to hyperuricaemia (Softic et al., 2020). Hyperuricaemia has been associated with raised blood pressure, body mass index (BMI), and triglycerides, as well as reduced HDL-C, decreased peripheral tissue insulin tolerance, and diabetes mellitus (Biradar et al., 2020). Excessive dietary fructose consumption is known to promote reactive oxygen species (ROS) accumulation. As well as the production of other various proinflammatory mediators such as interleukin (IL)-1β and IL-6, IL-12, tumour necrosis factor α (TNFα), and monocyte chemoattractant protein 1, which subsequently lead to cellular inflammation, oxidative stress and tissue damage (Jensen et al., 2018, Tamer et al., 2020). As noted above, many experimental studies have linked fructose metabolism to the development of multiple endocrine and metabolic disturbances. Which often result in the development of metabolic dysfunction, including NAFLD and NAFPD, due to increased ectopic lipid deposition.

1.3.3. Ectopic fat accumulation due to high fructose diets

Fructose is a powerful obesogenic agent, that may operate indirectly by delivering extra energy, leading excessive lipid accumulation, particularly in non classical fat storage organs (i.e. build up of triglycerides in their cytoplasm) such as the liver and pancreas. High fructose induced ectopic lipid deposition is closely linked to insulin resistance and hypertriglyceridemia and vice versa (Tamer et al., 2020).

The pathogenesis of high fructose induced hypertriglyceridemia can result from one of the following mechanisms or a combination of them all: a) High fructose consumption resulting in excessive production of triosephosphates, lactate, and fatty acids, leading to hypertriglyceridemia (Hannou et al., 2018, Tamer et al., 2020); b) Feeding with high fructose stimulating the expression of the key lipogenic enzymes (such as fatty acid synthase and acetylCoA carboxylase) via activation of transcription factors, carbohydrate-responsive element binding protein (ChREBP)1 (Koo et al., 2008, Iizuka, 2017), and sterol regulatory element binding protein1c (SREBP-1c), which eventually lead to hypertriglyceridemia by upregulating hepatic *de novo* lipogenesis (Haas et al., 2012); c) Additionally, high fructose intake can compound hypertriglyceridemia by decreasing hepatic fatty acid oxidation, decreasing hepatic VLDL secretion, and enhancing esterification of FFAs via reuptake of peripheral fatty acids from the adipocytes or diet (Softic et al., 2020).

Simultaneously, with hypertriglyceridemia, high fructose consumption can trigger both hepatic and peripheral insulin resistance via one or all of the mechanisms highlighted below; a) High fructose intake leads to stimulation of cellular stress responses by stimulating the production of ROS and/or inflammatory cytokines resulting in whole body insulin resistance (Du Toit and Donner, 2012); b) High fructose consumption can enhance hepatic *de novo* lipogenesis leading to hypertriglyceridemia, the build up of lipids (in the hepatocytes, visceral fat, and muscle), increased diacylglycerol, and ceramides, and eventually insulin resistance both in the liver and peripheral tissues (Tamer et al., 2020); c) High fructose consumption induced hyperuricaemia can secondarily cause endothelial cell dysfunction, impaired insulin induced vasodilatation. Consequently failure to increase muscle blood flow after a meal, leading to insulin resistance (Tappy, 2012). The above noted metabolic outcomes secondary to high fructose consumption resulting in lipid deposition in non adipose tissues lead to the development of NAFPD and

NAFLD (Ou et al., 2013, Bray and Popkin, 2014, Jensen et al., 2018). In the next section, NAFPD is discussed further.

1.4. Non-Alcoholic Fatty Pancreas Disease (NAFPD)

1.4.1. Definition of non-alcoholic fatty pancreas disease

Non-alcoholic fatty pancreas disease (NAFPD) refers to excessive fat deposition in the pancreatic tissue, which might progress to steatopancreatitis (pancreatic inflammation due to excessive fat deposition), fibrosis. That might eventually pancreatic cancer, in association with obesity and insignificant alcohol consumption (Chen et al., 2020b). Notably, significant alcohol intake, in relation to NAFPD, is defined as an intake of more than 30g of alcohol in men and more than 20g of alcohol in women on average per day (Yu and Wang, 2017). NAFPD is closely linked with failure of β cell function, insulin resistance, type 2 diabetes, severe acute pancreatitis, pancreaticoduodenal leakage, and NAFLD (Chen et al., 2020b, Psallas and Vasileiadis, 2020). The incidence of NAFPD is rising with the increasing cases of obesity globally, however, little is known about its prevalence worldwide (Yao et al., 2020).

1.4.2. Prevalence of non-alcoholic fatty pancreas disease

The epidemiology of NAFPD in the general population worldwide is unknown due to the lack of standard screening tools (Psallas and Vasileiadis, 2020). However, evidence shows that the occurrence of NAFPD differs largely based on the ethnicity of the population, and the methods used to diagnose the disease (Yu and Wang, 2017). Recent reports state that NAFPD is on the rise and its estimated prevalence in the general population is between 16 to 35% and about 67% in patients with NAFLD (Rosenblatt et al., 2019). Data concerning the prevalence of NAFPD in individual countries is very scanty, however, evidence from cross sectional studies in Indonesia, China, America, South Korea, and Taiwan, among adult individuals, revealed a diverse prevalence of NAFPD (Table 1.2) (Lesmana et al., 2015, Singha et al., 2017, Yu and Wang, 2017, Weng et al., 2018, Psallas and Vasileiadis, 2020). The prevalence of NAFPD in Africa remains largely unknown; however, studies in the United States of America showed that Hispanics accumulate more pancreatic fat than African Americans (Yu and Wang, 2017).

According to previous studies, the prevalence of NAPFD is higher in men compared to women before the age of 50 years, but the NAFPD prevalence appears to be

similar between postmenopausal women, and men (Paul and Shihaz, 2020). Although, most of the evidence available showed that the prevalence of NAFPD increases with advanced age, children are not excluded from it (Psallas and Vasileiadis, 2020). A retrospective study in America carried out among 232 hospitalized patients, aged 2 to18 years, found NAFPD in 10% of subjects using abdominal CT (Computed tomography) (Fu et al., 2017). Another hospital based study in South Korea found NAPFD in 58 out of 121 obese children (mean age 13.16 ± 2.69 years), who underwent abdominal ultrasound (Della Corte et al., 2015).

Table 1.2 The prevalence of NAFPD in some countries

Country of study	**Number of study subjects**	**Diagnostic Tool used**	**Prevalence of NAFPD**	**References**
Indonesia	1052, adults (720 Males and 334 females)	Abdominal ultrasound	(315) 35.0% (228 males and 87 females)	(Lesmana et al., 2015)
China (Yangzhou)	2093 adults (1443 male and 650 females)	Abdominal ultrasound	(56) 2.7% (28 male and 25 females)	(Wong and Chan, 2020)
China (Hong Kong)	922 adults (508 males and 414 females)	Magnetic resonance imaging (MRI)	(575) 16.1% (66 males and 44 females)	(Wong et al., 2014)
America	230 adults (102 males and 128 females)	Endoscopic ultrasound (EUS)	(64) 27.8% (27 males and 37 females)	(Sepe et al., 2011)
South Korea	284 adults (102 males and 182 females)	Endoscopic ultrasound (EUS)	(110) 38.7% (33 males and 77 females)	(Choi et al., 2010)

1.4.3. Risk factors and pathophysiology of non-alcoholic fatty pancreas disease

Numerous studies point out that NAFPD is closely correlated with hepatic steatosis, Increased visceral fat content, higher BMI, insulin resistance, metabolic syndrome (Chen et al., 2020b), diabetes, and dyslipidaemia (Rosenblatt et al., 2019, Paul and Shihaz, 2020). To date, no clear mechanisms (pathophysiology) have been identified that lead to the development of NAFPD. However, studies have hypothesized that

obesity leads to lipid accumulation in the pancreas in two stages: Firstly, intracellular deposition of triglycerides in the pancreatic cells from obesity induced hypertriglyceridemia, which occurs as a result of increased lipogenesis and reduced mitochondrial fatty acid beta oxidation, as well as an increase in peripheral lipids lipolysis (Coulson-Geissmann, 2018). Secondly, the increased pancreatic lipid deposition in the first stage, together with the lipid driven proinflammatory cytokines and adipokines, will in turn lead to pancreatic β cell dysfunction and β cell death (Aisyah and Siti, 2019).

The reduced insulin secretion due to the β cell death increases circulating FFAs (free fatty acids), and the lipid mediated inflammatory activities consequently lead to the development of a fatty pancreas (Yu and Wang, 2017, Aisyah and Siti, 2019). This excess fat can be deposited either in the interlobular spaces (fatty deposition in the extra/perilobular compartments) or intralobular spaces (fatty deposition within the pancreatic lobules or parenchyma). The latter is considered more troublesome since it is associated with neoplastic tendencies in the pancreas (Takahashi et al., 2018).

1.4.4. Diagnosis of non-alcoholic fatty pancreas disease

To date, there are no reliable haematological variables for the diagnosis of NAFPD (Pinte et al., 2019). However, radiological imaging (visualisation) tools such as Ultrasonography (USG), Computed tomography (CT), endoscopic ultrasound (EUS), and magnetic resonance imaging (MRI) are widely used to diagnose NAFPD (Paul and Shihaz, 2020). Some of the limitations associated with these tools include radiation exposure, they are very expensive, not readily available, require sedation, poor visibility of the pancreas due to obesity or bowel gas, and they cannot accurately predict pancreatic inflammation and fibrosis (Ballester-Vallésa et al., 2020, Paul and Shihaz, 2020). Pancreatic biopsy and histology are the most reliable tools for the diagnosis of NAFPD and remain the gold standard for the evaluation of NAFPD since they accurately define pancreatic fatty infiltration, inflammation, and fibrosis (Pinte et al., 2019, Vieira et al., 2020). Hence, pancreatic tissue biopsy and histology are regarded as the most important investigation tools in the diagnosis, staging, and evaluation of treatment in the management of patients with NAFPD (Pinte et al., 2019).

1.4.5. Management of non-alcoholic fatty pancreas disease

There are no approved guidelines for the treatment of NAFPD. Interestingly, NAFPD is a reversible condition in the early stages, which is achieved by modifying the risk factors associated with NAFPD (Aisyah and Siti, 2019). This can be done in two ways, with either non pharmacological or pharmacological therapies (Pinte et al., 2019). The non pharmacological measures involve weight reduction and lifestyle modifications. Such as the consumption of diets containing minimal or no fat and intensive exercise training, which have been shown to reduce pancreatic fat content and improve insulin sensitivity (Pinte et al., 2019, Pieńkowska et al., 2020).
Some of the pharmacological agents currently in use are metformin and troglitazone, angiotensin type1 receptor blockers, statins, and somatostatin analogues (such as Sandostatin). They have shown some promising results in the treatment of NAFPD (Pinte et al., 2019, Gomes-Porras et al., 2020). However, these drugs are not without significant side effects such as constipation, gastrointestinal discomfort, hepatitis, pancreatitis, erectile dysfunction, and increased risk of gallbladder stone formation (Wrobel et al., 2017, Gomes-Porras et al., 2020). Consequently, the need to find better treatment options for NAFPD and associated metabolic disorders such as insulin resistance, obesity, and NAFLD. In the next section, NAFLD will be discussed further.

1.5. Non-Alcoholic Fatty Liver Disease (NAFLD)

1.5.1. Definition of non-alcoholic fatty liver disease

NAFLD is a common universal cause of chronic hepatic injury, (Younossi et al., 2016, Wong and Chan, 2020). NAFLD comprises a series of hepatic diseases in individuals with significantly low or no history of alcohol ingestion (i.e. ≥ 30 g/day for males and 20 g/day for females) (Lin et al., 2020), and other causes of liver disease. NAFLD can progress from simple hepatic steatosis (i.e. presence of > 5% of fat of the total hepatocytes) to non-alcoholic steatohepatitis (NASH), which may eventually culminate in to liver cirrhosis, fibrosis, and hepatocellular carcinoma (Xu et al., 2019, Zhang et al., 2020).

1.5.2. Prevalence of non-alcoholic fatty liver disease

Generally, it is has been reported that approximately 25% of the general population are living with NAFLD and the prevalence is rapidly increasing globally (Wong and Chan, 2020). However, the prevalence of NAFLD differs across continents, with the Middle East having the highest prevalence (31.8%), followed by South America (30.4%), Asia (27.4%), North America (24.1%), Europe (23.7%) and Africa with the least of all (13.5%) (Younossi et al., 2016, Araujo et al., 2018, Iqbal et al., 2019, Wu et al., 2020). A study on NAFLD among 233 overweight/obese subjects in the Western Cape of South Africa by Kruger et al. (2010), found 182 subjects (78%) with NAFLD (with the aid of abdominal ultrasound) and 111 (87%) out of 127 subjects (among the 233 patients who consented to liver biopsy) with NAFLD (liver biopsy proven). Out of those with NAFLD 46 subjects (36%) had NASH (non-alcoholic steatohepatitis) (Kruger et al., 2010). Studies in Nigeria and Sudan reported a NAFLD prevalence of between 9.5 to 16.7% in individuals with diabetes and between 1.2 to 4.5% in individuals without diabetes (Olusanya et al., 2016, Younossi, 2019).

The development of NAFLD is synchronous to that of type 2 diabetes, obesity and metabolic syndrome in humans, the occurrence of NAFLD in very obese individuals can reach more than 90% (Lee et al., 2019). Despite a strong association between NAFLD and obesity, NAFLD can also be diagnosed in individuals with a normal BMI (Iqbal et al., 2019). Shi et al. (2020) reported a NAFLD prevalence of 16% and 10% in non obese and lean populations, respectively. Sex is another significant factor in the prevalence of NAFLD; studies have shown that NAFLD is more commonly seen in males than females (Yu et al., 2019b). In Hong Kong China, a community based study involving 264 obese and overweight subjects (aged 19 to 72 years), identified the overall prevalence of NAFLD to be 28.6% (37% in men and 23% in women) using magnetic resonance spectroscopy (Wong et al., 2012). Another, study in Japan reported the prevalence of NAFLD to be 17.7% in women and 41.0% in men (Eguchi et al., 2012).

Age differences are another crucial factor that influences the prevalence of NAFLD. A cross sectional study in Tokyo Japan, involving 977 male and 1467 female participants, with an age range of 40 to 69 years, identified the prevalence of NAFLD to be 6.8% and 7.5% in the participants with the mean age of 49.1 and 57.6 years, respectively (Tajima et al., 2019a). Similarly, a longitudinal study in the Asia Pacific

region observed an increase in the prevalence of NAFLD in older subjects compared to younger subjects (Estes et al., 2020). Children are also affected by NAFLD, with a prevalence of 26.0% (29.4% in males and 22.6% in females) among 408 obese children (aged 9 to17 years), who underwent abdominal MRI in a hospital based study in America (Yu et al., 2019a). Genetic predisposition is also another significant factor in the development of NAFLD. Different genetic loci, such as Patatin like phospholipase domain containing 3, Protein phosphatase 1 regulatory subunit 3B, Neurocan, Glucokinase regulator, and Transmembrane 6 superfamily member 2, has been connected with increased risk or susceptibility to having NAFLD (Simoes et al., 2018, Shousha et al., 2020, Walker et al., 2020).

The prevalence of NAFLD is also more common in urban populations compared to rural populations; this could be due to dietary habits and lifestyles. For example, the prevalence of NAFLD in a rural population of western Bengal in India was identified to be 8.7%, compared to urban areas where the NAFLD prevalence's were reported to be 16.6%, 24.5%, and 32% in western, eastern, and southern cities of Indian respectively (Wong and Chan, 2020, Wu et al., 2020). NAFLD is a heterogeneous disease that affects all genders, age groups, ethnicity/race, and geographical locations. Moreover, some individuals with certain health conditions are at higher risk of developing NAFLD than others.

1.5.3. Risk factors and pathophysiology of non-alcoholic fatty liver disease

1.5.3.1. *Risk factors of non-alcoholic fatty liver disease*

Obesity, diabetes mellitus and insulin resistance are among the strongest predisposing factors related to the development of NAFLD (Mirea et al., 2020), as well as sedentary lifestyles, ingestion of diets rich in fat and/or refined sugars (as HFCS in processed foods and beverages) and dyslipidaemia (Padma and Sankar, 2020). Advanced age and certain genetic variants are also associated with an increased likelihood of developing NAFLD, as highlighted above (Ishizuka et al., 2020, Shousha et al., 2020). Taking into consideration the risk factors, it's very important to properly understand the pathogenesis of NAFLD, in order to instil proper preventive and curative measures for treating the vulnerable individuals in our society.

1.5.3.2. *Pathophysiology of non-alcoholic fatty liver disease*

NAFLD is complex in nature and extremely heterogeneous among various individuals (Yu et al., 2019b). The pathogenesis of NAFLD has been previously described by the 'two hits' hypothesis (initial development of hepatic steatosis being the first hit followed by oxidative stress from inflammatory cytokines and adipokines, which may then lead to NASH and fibrosis as a second hit). This hypothesis later found to be too simplistic to define the actual mechanisms, molecular and metabolic changes involved in the aetiology of NAFLD (Angulo, 2002, Schreuder et al., 2008). The more recent proposal of a 'multiple hit' theory, which is regarded as more accurate in defining the pathogenesis of NAFLD has been proposed. The theory considers several insults working simultaneously in susceptible individuals to bring about NAFLD. Some of these insults include but are not limited to genetic factors, hormones secreted by adipose tissue, gut microbiota dysbiosis, insulin resistance, and dietary factors such as increased dietary fructose consumption (Porrasa et al., 2017, Flisiak-Jackiewicz and Lebensztejn, 2019, Ullah et al., 2019, Yu et al., 2019b). High fructose consumption is linked to the aetiopathogenesis of NAFLD in many ways, which include: a) adipose tissue dysfunction which in turn causes the release of proinflammatory cytokines, adipose tissue lipolysis, adipose tissue insulin resistance, increased fatty acid uptake by the liver and eventually hepatic steatosis; b) hepatic insulin resistance due to hepatic lipid deposition particularly DAG, which will, in turn, stimulate gluconeogenesis and hepatic *denovo* lipogenesis via activation of SREBP1 and ChREBP1, compounding the hepatic steatosis; c) mitochondrial dysfunction due to endoplasmic reticulum stress, resulting from the hepatic steatosis which will further exacerbate the hepatic steatosis via inhibition of hepatic lipids export (Chalasani and Szabo, 2016, Bessone et al., 2019). These mechanisms will create a vicious cycle that might eventually lead to the progression of simple steatosis to hepatocellular carcinoma or end stage liver failure if untreated (Pai et al., 2019, Tamer et al., 2020).

Hence the needs for early, proper diagnosis and interventions in order to reverse, slow, or prevent the progression of simple steatosis into more advanced irreversible disease, which is associated with huge clinical and economical burdens to the patients, their families, and society (Fuchs, 2019, Thanapirom and Tsochatzis, 2019, Yoo et al., 2019). Therefore, the next section will concentrate on the diagnosis of NAFLD.

1.5.3.3. *Diagnosis of non-alcoholic fatty liver disease*

Diagnosing NAFLD requires the exclusion of other causes of fatty liver by proper evaluation of historical and clinical features and other possible causes of hepatic fat deposition such as alcoholic liver disease (Barshop et al., 2008). Several radiological (imaging) tools, biochemical parameters, and histological criteria used in diagnosing NAFLD rely on the presence (> 5%) of lipid droplets in the hepatocytes (Shima et al., 2020).

Radiological tools are non invasive imaging techniques used to directly view the liver and assess the level of hepatic steatosis subjectively by comparing the liver echogenicity with the echogenicity of other abdominal organs like the kidneys or pancreas. Some of these tools include USG, CT scan, and MRI. However, the tools are limited because they cannot detect inflammation, fibrosis, and the early stages of NAFLD (Chuah et al., 2020, Leong et al., 2020). Hence, the need to use other complementary diagnostic tools that employed plasma levels of certain biochemical parameters that are considered to be surrogate biomarkers of liver function. Elevated serum levels of liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Are linked to hepatic steatosis, and considered to be indicators of impaired liver function or liver damage, as well as predictors of NAFLD (Rotman and Kapuria, 2020). However, they are also limited in their diagnostic value because of their low specificity and sensitivity (other liver diseases different from NAFLD are also associated with elevated serum concentrations of liver enzymes) (Rotman and Kapuria, 2020). Routine biochemical parameters, although not specific to NAFLD alone, can still offer valuable information, because they can serve as indicators of an altered metabolic condition, particularly as related to fructose and lipid metabolism, which are core to the development of NAFLD. Some of these include, but are not limited to serum fasting blood glucose (FBG), fasting insulin levels, triglycerides, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total protein, total bilirubin and albumin, uric acid level (Castera, 2020, Kaya et al., 2020).

Other biochemical parameters that are also used as diagnostic indicators of NAFLD include hormones that are involved in lipid metabolism such as fibroblast growth factor 21, leptin, resistin, and adiponectin. Lower serum concentrations of adiponectin and increased serum levels of fibroblast growth factor 21, leptin, and resistin have been associated with NAFLD. However, these biochemical parameters

are also associated with limitations such as poor sensitivity and specificity, they are expensive to measure, and are not yet ready for routine clinical application (Tucker et al., 2020).

NAFLD has been linked with elevated serum levels of biochemical markers of inflammation such as tumour necrosis factor (TNF), interleukin-6 (IL-6), IL-8, and C-reactive protein (Baselli et al., 2020). However, these biochemical markers have low specificity and sensitivity in the diagnosis of NAFLD, because increased serum concentrations of these markers are also seen in other metabolic diseases like diabetes mellitus and obesity (Lee et al., 2019, Baselli et al., 2020). The use of biochemical markers of liver apoptosis and necrosis such as cytokeratin 18 is gaining attention. Elevated serum level of cytokeratin 18, which is the intermediate filament protein released by the dead liver cells, has been linked to NAFLD. However, the use of cytokeratin 18 is premature and needs further research (Takahashi et al., 2018).

There have been attempts to employ non invasive tools that use combinations of biochemical markers and clinical parameters to predict inflammation and fibrosis in patients with NAFLD. Some of these tools include AST/ALT ratio, AST/platelet ratio index (APRI), BARD score (which includes BMI, AST/ALT ratio, and presence or absence of diabetes or impaired fasting glucose) (Marjot et al., 2020). NAFLD Fibrosis Score (NFS), which is based on platelet count; albumin concentration; BMI, and the presence or absence of diabetes or impaired fasting glucose (Kaya et al., 2020). Fibrosis4 index (FIB-4) which consists of age and platelet count (Kaya et al., 2020). Limitations of these tools include their incomplete validation, high cost, unavailability or inaccessibility for routine clinical application, low specificity, and sensitivity and lower accuracy compared to liver biopsy in confirming the early stages of NAFLD (Castera, 2020, Kaya et al., 2020, Robinson and Wong, 2020).

Liver biopsy is the most reliable tool for the diagnosis, staging, and monitoring treatment and progress of NAFLD because it doesn't only show fatty accumulation in the liver, it also shows evidence of inflammation and fibrosis, as well as the distribution of the fat in the liver and, is hence it is regarded as the gold standard investigation for diagnosis of NAFLD (Iqbal et al., 2019, Rotman and Kapuria, 2020). There are many scoring systems that are employed for semi quantitative evaluation of liver biopsy and histology; one of the widely used methods is the NAFLD activity score (NAS) with fibrosis scores (Santiago-Rolón et al., 2016). That grades the hepatic steatosis, lobular inflammation, ballooning and fibrosis as described later in

section 2.15.3 and section 2.15.4. Following diagnosis and staging of NAFLD, several treatment strategies described below may be used either alone or in combination, to prevent, treat, or slow the progression of NAFLD.

1.5.4. Management of non-alcoholic fatty liver disease

Current standard therapeutic guidelines for managing NAFLD patients do not exist (Thanapirom and Tsochatzis, 2019). However, many studies have found lifestyle modifications; some pharmacological agents as well as some surgical procedures can offer tremendous benefits. The majority of these studies, if not all, focused on the treatment of persons with NAFLD by modifying the risk factors, for example, the dyslipidaemia, diabetes, obesity, and insulin resistance (Barshop et al., 2008, Chalasani et al., 2012, Carulli et al., 2013, Padma and Sankar, 2020, Salman et al., 2020a). The management of NAFLD can therefore be divided into (i) lifestyle modifications (ii) pharmacological therapy and (iii) surgical intervention.

There is a plethora of evidence from many studies, that lifestyle modification which includes increased physical exercise and dietary modifications (consumption of low calorie, high fibre diets and avoiding processed foods rich in refined sugars and fat).Reduced hepatic lipid content, insulin resistance, hyperglycaemia, improved hepatic histology (Padma and Sankar, 2020, Vittorio and Lavine, 2020). Initial treatment measures for early NAFLD involve dietary and lifestyle modifications as mentioned above, particularly in patients with early disease. However, in advanced NAFLD, lifestyle modification measures are used together with pharmacotherapy and/or surgical interventions (Rotman and Kapuria, 2020).

Surgical interventions such as bariatric surgical procedures, for example, laparoscopic sleeve gastrectomy and Roux-en-Y gastric bypass are gaining popularity because of their role in weight reduction. These bariatric surgical procedures are used in the treatment of morbid obesity and NAFLD. Have proven to improve obesity, biochemical parameters of NAFLD, hepatic steatosis, as well as steatohepatitis, and fibrosis (Endo et al., 2019, Salman et al., 2020a, Salman et al., 2020b). Liver transplant is another surgical procedure available when the above surgical interventions fail to reverse NAFLD, though it is not routinely used in the treatment of NAFLD (Cananzi et al., 2019). However these procedures are not without limitations i.e. they are very expensive, have limited use in the treatment of NAFLD in general populations. Due to the low availability of liver donors, and

complications related to surgery and possible transplant rejection are not uncommon (De Almeida et al., 2006, Yu et al., 2019b).

Many pharmacological agents approved for use in the management of other metabolic diseases, such as diabetes mellitus and obesity, have shown positive results in treating NAFLD. Some of these medications include but are not limited to; antidiabetic agents such as biguanide (e.g. metformin), glucagon like peptide 1 receptor agonists (e.g. liraglutide), and thiazolidinediones (e.g. rosiglitazone and pioglitazone) (Rotman and Kapuria, 2020). These drugs have been shown to improve NAFLD in several clinical trials by improving hepatic lipid content, via suppressing hepatic de novo insulin sensitivity, increasing mitochondrial β oxidation, and encouraging weight loss in NAFLD patients (Iogna Prat and Tsochatzis, 2018, Rotman and Kapuria, 2020).

Lipid lowering agents like fibrates (gemfibrozil) and statins (simvastatin and atorvastatin) are other drugs used to treat NAFLD patients and have been shown to reduce hepatic lipid content by decreasing hypertriglyceridemia and hypercholesterolemia (Van Den Berg et al., 2019, Zhang et al., 2019). Antioxidants such as vitamin C, vitamin E, and pentoxyfilline have been used to mitigate the progression of hepatic steatosis and hepatocellular damage secondary to inflammation, oxidative stress, and damage due to reactive oxygen species in NAFLD (Du et al., 2014, Ivancovsky-Wajcman et al., 2019). Ursodeoxycholic acid, an epimer of chenodeoxycholic acid (endogenous bile acids) has been shown to prevent hepatic liver injury, hepatocytes apoptosis, and fatty degeneration due to their cytoprotective, membrane stabilizing, and anti-apoptotic effects (Gheibi et al., 2019). Other pharmacological agents such as acetyl-CoA carboxylase enzyme inhibitor, diacylgylycerol, O-acyltransferases inhibitor, farnesoid X receptor agonists, and omega-3 fatty acid have been found to have significant anti-NAFLD effects (Mclaren et al., 2018, Van De Wiel et al., 2019, Valenzuela and Videla, 2020, Xi and Li, 2020).

Although, the synthetic drugs used in management of NAFLD might offer a few beneficial effects, they are also associated with significant unwanted side effects such as indigestion, muscle pain and difficulty sleeping. They are also costly and not readily available or accessible in both developing and underdeveloped countries such as India and Niger. Plant sourced drugs (phytochemicals) have a natural origin, ample natural sources, are simple to use, inexpensive, have a holistic nature of therapy, and perceived reduced side effects. For decades the plant sourced

medicines have been used in the treatment of chronic disorders, both in developed and developing countries. Scientists are giving more attention to finding/developing more suitable agents, particularly phytochemicals, for the treatment of NAFLD and NAFPD (Yao et al., 2016, Bule et al., 2019). Quercetin is one such phytochemical, with proven pharmaceutical benefits, that have improved many acute and chronic diseases as detailed below (Nimrouzi et al., 2020). So far, little is known about the use of phytochemicals (such as quercetin) in the management of NAFLD and NAFPD.

1.6. Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a common plant flavonoid, which is widely distributed in human dietary sources (Tabrizi et al., 2019). Quercetin has been shown to have several health beneficial effects which are discussed later. Quercetin has been used for decades as a therapeutic agent to prevent and treat several chronic diseases (Zhao et al., 2017, Lesjak et al., 2018).

1.6.1. Natural sources of quercetin

Quercetin is found in abundance mostly in the leaves and the outer parts of edible plants such as vegetables (onions, peppers, asparagus, lettuce, spinach, broccoli), fruits (apples, strawberry, grapes, cherries, cranberries, raspberry, cloudberry), teas (black tea, green tea, fennel, tea) and other foods items such as red wine and green beans (Lesjak et al., 2018, Cione et al., 2019, Fuentes et al., 2020).

1.6.2. Physical and chemical properties of quercetin

Quercetin, a yellow crystalloid, is not soluble in cold water, poorly soluble in hot water, soluble in lipids, acetone pyridine, acetic acid, and very soluble in ether or methanol (Kumar et al., 2017, Magar and Sohng, 2020). Quercetin is only synthesized by plants and occurs mainly in two forms namely glycosides (rutin) and aglycones (without sugar) (Magar and Sohng, 2020). Quercetin, like other flavonoids, is made up of a 15 carbon atoms diphenyl propane structure. It has a closed heterocyclic pyran ring in addition to two benzene rings, with five hydroxyls groups placed at different positions (3,3',4',5,7) (Figure 1.1). Substituting the hyroxyl group at position 3 with a sugar moiety (galactose, glucose, and rhamnose) will

result in quercetin glycoside formation which makes quercetin more soluble in water and increases absorption (Khan et al., 2020, Magar and Sohng, 2020).

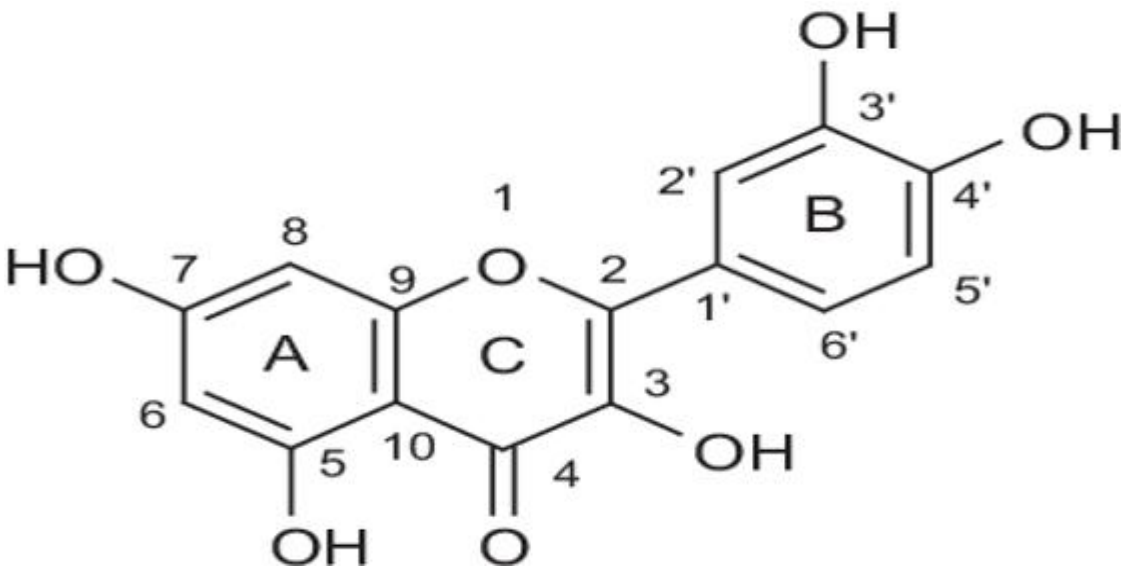

Figure 1.1 Arrangement of chemical bonds and molecular composition of quercetin (3, 3',4',5,7-pentahydroxyflavone) (Magar and Sohng, 2020).

1.6.3. Absorption, metabolism, and excretion of quercetin

Quercetin can be administered orally since many studies have suggested that quercetin absorption occurs in all parts of the gastrointestinal tract, from the stomach to the colon (Li et al., 2016, Mansoorian et al., 2019). Absorption of quercetin can be augmented by administering quercetin in combination with a high fat (17%) diet, non-digestible oligosaccharides, Bromelain (an enzyme derived from pineapple), or alcohol (Almeida et al., 2018, Mansoorian et al., 2019, Wang et al., 2020). Following, oral administration of quercetin, quercetin absorption occurs in two different ways into the enterocytes, firstly by passive diffusion into the enterocytes following deglycosylation of quercetin glycosides by the luminal hydrolase enzymes. Secondly, transportation of quercetin glycosides into enterocytes occurs via a transporter followed by deglycosylation by the cytosolic glycosidase in the enterocyte (Almeida et al., 2018, Lesjak et al., 2018).

Following absorption, quercetin then undergoes metabolism and biotransformation in the liver, kidneys, and small intestine where it is glucuronidated, methylated or sulphated before distribution in various tissues (Li et al., 2016, Almeida et al., 2018).

In plasma, almost 99.4% of quercetin is conjugated with plasma proteins, which could be the reason for its low bioavailability to cells (Li et al., 2016). The biological half-life of quercetin on average is 3.5 hours (Ferenczyova et al., 2020a), after which it is eliminated in the urine, stool, and exhaled air (Li et al., 2016, Ferenczyova et al., 2020b). Quercetin is considered to have a wide safety profile, since quercetin is well tolerated following very high doses of up to 250 mg/day; hence, quercetin is considered to be generally safe. Dietary enrichment with quercetin has been approved in many countries, due to its prophylactic and therapeutic effects against a wide range of diseases as detailed in the next section (Andres et al., 2018, Ferenczyova et al., 2020a).

1.6.4. Therapeutic effects of quercetin

1.6.4.1. *Hepatoprotective properties of quercetin*

Orally administered quercetin has been shown to attenuate liver damage, secondary to high dietary fat induced inflammation, and oxidative stress (Xu et al., 2019). Donaldson and colleagues reported a 50% reduction in hepatic fat at the end of 8 weeks of feeding weanling, female, Sprague Dawley rats, a high fat, high fructose diet and quercetin (75 mg/kg body weight/ day) (Donaldson et al., 2019). Likewise, in a study by Ying et al. (2013), reported improved hepatic steatosis and morphological features of NAFLD by attenuating lipid metabolism and inflammation in male gerbils fed a high fat diet and orally administered quercetin (30 and 60 mg/kg body weight/day for 14 day).

Quercetin has been demonstrated to have strong hepatoprotective effects against hepatic damage caused by hepatic steatosis (Miltonprabu et al., 2017), drug (isoniazid) induced hepatic toxicity (Liua et al., 2020), ethanol induced hepatic damage (Pingili et al., 2020), chemical (carbon tetrachloride) induced liver damage (Sa'id et al., 2020) and metallic induced liver damage. Via its powerful metal chelating, free radical scavenging and antioxidant activities (Miltonprabu et al., 2017). These powerful antioxidant activities of quercetin have made it useful in treating many chronic diseases associated with increased oxidative stress such as hyperglycaemia and diabetes (Bule et al., 2019). Despite, the fact that many studies previously investigated the use of quercetin as a potential liver protector against several toxic insults, these were mainly in adults with a bias to males and not in growing female rats. There is also a dearth of studies on quercetin and high fructose

induced NAFLD and NAFPD in growing female rats as well as their associated risk factors such as obesity and diabetes.

1.6.4.2. *Hypoglycemic and anti-diabetic properties of quercetin*

Both hypoglycaemic and anti-diabetic effects of quercetin were observed in rat models of juvenile and adult onset diabetes mellitus (Bule et al., 2019). Dietary quercetin supplementation at various doses (25, 50, and 75 mg/kg body weight/day) for four weeks was able to significantly improve diabetes, by improving plasma insulin, hyperglycaemia, and glucosuria in an streptozotocin induced diabetic rat model (Srinivasan et al., 2018). Another study on diabetic Wistar rats treated with oral quercetin reported that quercetin supplementation at a dose of 0.02 mmol/kg body weight/ day, as an aqueous suspension 0.2 mmol % for 4 weeks, not only improved hyperglycaemia but also improved the hyperlipidemia associated with diabetes by decreasing total cholesterol and triglycerides levels in diabetic rats (Velescu et al., 2017).

Similarly, daily dietary quercetin (50 mg/kg body weight) treatment for 28 days in a type 2 diabetic rat model was reported to improve hyperglycaemia, insulin sensitivity, as well as hyperlipidaemia (Abdelkarem and Fadda, 2017). Hence, the use of quercetin as a potential hypolipidaemic and anti obesity agent is detailed in the next section.

1.6.4.3. *Hypolipidaemic and anti-obesity properties of quercetin*

Obesity and hyperlipidaemia have been closely linked to fat deposition in non-fat organs like the liver and pancreas, as seen in NAFLD and NAFPD (Estes et al., 2020, Paul and Shihaz, 2020). There are many studies that have reported positive effects of quercetin in fat metabolism. For example, a Korean study with overweight and obese participants reported significant reduction in body fat and overall BMI following 12 weeks of orally administered quercetin (100 mg/day/subject) (Lee et al., 2016).

Nettore and his co-workers reported quercetin administration at 0.26 mg/kg body weight/day, for 84 days in male Wistar rats, was able to reduce adipocyte size, body weight, and white adipose tissue by reducing the activities of the two main adipogenic genes (i.e. Peroxisome proliferator activated receptor gamma (PPARγ) and enhancer-binding protein alpha). They also showed that quercetin was able to

improve the dyslipidaemia and hepatic steatosis by activating the expression of beta-oxidation related genes such as Acyl-CoA synthetase-1 and Carnitine palmitoyl transferase 1 alpha (Nettore et al., 2019). Another study on seven weeks old male Sprague Dawley rats using orally administered quercetin at a dose of 0.1% of the high fat diet for 12 weeks, found that quercetin reduced serum LDL-C, triacylglycerol, and insulin. The same study also highlighted quercetin's ability to reduce TNF-α, hepatic lipid deposition, and modulate the structure and diversity of the gut microbiota associated with obesity and NAFLD (Peng et al., 2020).

1.6.4.4. *Antimicrobial properties of quercetin*

Human and animal based experimental models of obesity, diabetes, and NAFLD were observed to have gut microbiota dysbiosis (Aron-Wisnewsky et al., 2020). Previous *in vivo* and *in vitro* studies have demonstrated quercetin's antimicrobial activity achieved through the restraint of the growth of many microbes such as *Salmonella typhi, Escherichia coli, Enterococcus faecalis, Bacillus subtilisin, Bacillus cereus* (Prabhakaran et al., 2020, Yadav et al., 2020), *Pseudomonas aeruginosa, Staphylococcus aureus, Shigella flexneri,* and *Proteus vulgaris* (Jaisinghani, 2017). Quercetin's antimicrobial potential has been used both in animals and humans to restore the gut microbiota dysbiosis associated with obesity, diabetes mellitus, and NAFLD with a concomitant improvement in obesity, diabetes, and NAFLD symptoms (Porras et al., 2019), thereby preventing metabolic and cardiovascular complications associated with obesity and diabetes mellitus, like hypertension.

1.6.4.5. *Anti-hypertensive and cardio-protective properties of quercetin*

NAFLD is regarded as an important predisposing factor for the development of many illnesses related to the circulatory system such as myocardial infarction, cardiomyopathy, hypertension, and stroke (Labenz et al., 2020).
Dietary quercetin supplementation at 20 mg/kg/day for 42 days to obese Zucker diabetic fatty rats has been found to improve vascular function and prevent the development of hypertension (Ferenczyova et al., 2020b). Similarly, the antihypertensive effect of quercetin has been reported in humans, in a meta-analysis of dietary quercetin supplementation of 8 weeks or more in humans, involving seventeen trials with 896 participants reported that quercetin significantly decreased both systolic and diastolic blood pressure in humans (Huang et al., 2020).

Another animal study used dietary quercetin supplementation at 80 mg/kg/day for 14 days in male Sprague Dawley rats and reported quercetin's potential to prevent acute myocardial infarction, via anti inflammatory and anti apoptotic effects and through the down regulation of the toll like receptor 4-nuclear factor signalling pathway (Ma et al., 2018). Additionally, Duarte and co-workers administered daily dietary quercetin of 10 mg/kg body mass for 5 weeks in adult male spontaneously hypertensive rat models and observed lowered blood pressure, decreased cardiac hypertrophy, and functional vascular changes and attributed these effects to the anti-oxidant activities of quercetin (Duarte et al., 2001).

1.6.4.6. *Anti-oxidant properties of quercetin*

Increased cellular inflammation, oxidative stress, and concurrent exhaustion of intrinsic antioxidant defensive mechanisms such as non enzymatic (e.g. glutathione, ascorbic acid, and vitamin E) and enzymatic (e.g. superoxide dismutase, catalase, and glutathione peroxidase), have been closely linked to the initiation and progression of ectopic lipid deposition. Like the one seen in NAFPD and NAFLD (Negri et al., 2020, Nimrouzi et al., 2020). Quercetin is regarded as a very powerful antioxidant agent because of its potential to enhance cellular antioxidant activities and the overall homeostatic oxidative balance, by improving both non enzymatic and enzymatic intrinsic antioxidant defence systems (Castañeda-Arriaga et al., 2020, Pingili et al., 2020).

Quercetin has been shown to improve non enzymatic intrinsic antioxidant defence system via amplification of cellular free radicals scavenging activities, as well as systemic glutathione concentration (Boots et al., 2008). Furthermore, quercetin enhances the enzymatic intrinsic antioxidant defence system as well, by enhancing the genes expression of enzymes responsible for cellular antioxidant defence system such as superoxide dismutase, catalase, and glutathione peroxidase (Bule et al., 2019, Boots et al., 2020). These antioxidant effects of quercetin have been exploited in studies with diabetic induced NAFLD and NAFPD rat models to reduce lipid deposition and /or the progression of simple steatosis to advanced disease such as pancreatic cancer and hepatocellular cancer in NAFPD and NAFLD, respectively (Omar et al., 2016, Yang et al., 2019).

1.6.4.7. *Anti-cancer properties of quercetin*

Studies have shown simple liver and pancreatic steatosis might progress and lead to the development of hepatocellular cancer and pancreatic cancer (Dite et al., 2020, Marjot et al., 2020). On their own NAFLD and NAFPD are considered as emerging risk factors for other gastro-intestinal tract tumors/cancers (Divella et al., 2019, Dite et al., 2020).

Similarly, *in vitro* and *in vivo* studies on the impact of quercetin on hepatocellular carcinoma have shown that quercetin was able to reduce tumor size by inducing and facilitating autophagy and apoptosis of the tumor cells, via up-regulation of Mitogen-activated protein kinase signalling pathways and down regulation of Protein kinase B/mammalian target of rapamycin pathway (Ji et al., 2019). Quercetin in combination with gemcitabine was also reported to facilitate apoptosis and autophagy in human pancreatic cancer cells, through inhibition of advanced glycation end products, and simultaneously increased chemosensitivity of gemcitabine (Lan et al., 2019). Quercetin has been used to facilitate cell death in the treatment of other neoplastic conditions such as breast cancer (Choi et al., 2008), colorectal cancer (Richter et al., 1999), and thyroid cancer (Celano et al., 2020).

1.6.4.8. *Other health benefits of quercetin*

Many studies have used or have recommended the use of quercetin for the treatment of different medical conditions such as wounds (Ajmal et al., 2019), hypertrophic scars and keloids (Moravvej et al., 2019), hyperuricaemia and gout (Ahmada et al., 2008), fungal infections (Rocha et al., 2019), drugs toxicity (Liua et al., 2020, Mahmoud et al., 2020) Liua et al., 2020). Allergic conjunctivitis (Ding et al., 2020), allergic asthma (Ravikumar and Kavitha, 2020), and infertility (Seda Karabulut et al., 2020, Tabrizi et al., 2020) are further targets for medicinal interventions with quercetin.

1.7. Justification of the Study

Various studies have confirmed that excessive fructose consumption is responsible for the current global epidemics of metabolic syndrome and its components, as well as the increasing prevalence of NAFLD and NAFPD. These metabolic disorders affect children, adolescents, and the elderly, of all sexes and races, hence, posing a global public health challenge because there are no approved guidelines for the

management of these disorders (Barshop et al., 2008, Chalasani et al., 2012). Some of the available current treatment options include weight loss via exercise and other lifestyle modifications (Lazo et al., 2010).

Pharmacological agents such as metformin, pioglitazone, statins, liraglutide, and fibrates, are also currently being used for the treatment of these metabolic disorders (Iogna Prat and Tsochatzis, 2018). However, these drugs tend to be mono-therapeutic and have significant adverse side effects, hence, novel treatment candidates with high efficacy, targeting multiple risk factors for metabolic disorders, and minimal side effects are needed for the treatment of these diseases.

Currently, a large percentage of the world's population relies on medicinal plants for their health care (Bule et al., 2019). A lot of these plants need to be scientifically validated for their claimed medicinal properties and the active ingredients need to be isolated. Phytochemicals are being targeted for potential use in the prevention and treatment of NAFLD and NAFPD, due to their natural origin, holistic nature of therapy and, perceived reduced side effects (Yao et al., 2016, Bule et al., 2019). Information on the use of phytochemicals in the management of the above mentioned metabolic disorders is lacking. Despite the sexually dimorphic presentation of the metabolic disorders, evidence available indicates that the bulk of the studies in this area have made use of adult, male rats. Consequently, the current study made use of growing female rats that received a high fructose diet to stimulate the development of NAFLD and NAFPD, to investigate the prophylactic ability of quercetin against these metabolic disorders.

1.8. Aim and objectives of the Study

The primary goal of the current study was to explore the ability of orally administered quercetin (post weaning) to prevent the development of NAFLD and NAFPD, as well as the metabolic dysfunction associated with a high fructose diet in weanling female Sprague Dawley rats. The objectives of the research were to determine the effects of orally administered quercetin and high fructose diet feeding in female Sprague Dawley rats from the early post weaning period, on the:

- development of metabolic dysfunction through the assessment of general impairment of metabolism by determining:

    - Circulating levels of metabolites (Fasting blood glucose, triglycerides, total cholesterol (TC) and high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C).
    - Adiposity (visceral and retroperitoneal fat pad mass).
    - Concentrations of hormones involved in metabolic regulation (insulin and adiponectin).
- development of non-alcoholic fatty liver disease specifically considering:
    - Hepatic lipid deposition and the development of hepatic steatosis.
- development of non-alcoholic fatty pancreas disease, specifically considering:
    - Pancreatic lipid deposition and the development of pancreatic steatosis.
- general health profile of the rats through the evaluation of:
    - Biochemical (surrogate) markers of liver function (alanine transaminase (ALT), total bilirubin, and albumin).
    - Biochemical markers of pancreatic function (amylase and lipase).
    - Biochemical markers of renal function (blood urea nitrogen and plasma creatinine).
    - Serum clinical metabolic markers of health (total protein, calcium, phosphate, uric acid, and globulin).
    - Red blood cell indices (packed cell volume (PCV), haemoglobin (Hb), mean corpuscular haemoglobin concentration (MCHC)).

1.9. Hypotheses

The null hypothesis (H_0) and the alternate hypothesis (H_1) for the study were:

- **H0:** Administration of quercetin to weanling female Sprague Dawley rats on a high fructose diet does not protect them from developing diet induced metabolic dysfunctions (including non-alcoholic fatty liver disease and non-alcoholic fatty pancreatic disease).
- **H1:** Administration of quercetin to weanling female Sprague Dawley rats on a high fructose diet protects them from developing diet induced metabolic dysfunctions (including non-alcoholic fatty liver disease and non-alcoholic fatty pancreatic disease).

CHAPTER 2: MATERIALS AND METHODS

2.1. Ethical Clearance for the Study

All the procedures and experiments were conducted in accordance with the protocol as approved by the Animal Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa. The committee was previously known as the Animal Ethics Screening Committee. For this study, the ethical clearance number is 2017/02/07B, (Appendix A and Appendix B for the certificates).

2.2. Animals, Housing and General Care

2.2.1. Animals

Thirty seven, 21 days old weanling female Sprague Dawley rat pups obtained from the Central Animal Service of the University of the Witwatersrand, with litters consisting of 8 to12 pups, were used in the study. The rats were housed at the rodent unit of the Central Animal Service. The rats were then allowed a three day acclimatisation period before starting the experimental interventions.

2.2.2. Housing and general care of the animals

The rats were housed individually in transparent polycarbon cages, with wood shavings as bedding and shredded paper was placed in the cages for the rats to nest in. For further environmental enrichment, sections of large diameter polyvinyl chloride pipes were also placed in the cages for the rats to tunnel in. Room temperature was kept at 25 ± 2°C, with a 12 hours light/dark cycle (with lights off between 7pm to 7am). Adequate ventilation was maintained in the animal holding facility throughout the acclimatisation and study periods. Rat cages were cleaned and the bedding was replaced twice weekly throughout the study period by the student with assistance of the animal attendants/technicians.

2.3. Study Design and Diet Treatment Groups

Figure 2.1 shows a diagrammatic representation of the study design. Thirty seven, 21 days old female Sprague Dawley rats were randomly assigned to one of four experimental groups, with 9 to10 rats in each group.

The first group (n=10) (C), was the negative control group and received commercially sourced standard rat chow (SRC) (LabChef, North West University/RB2005), tap water to drink, and plain gelatine cubes daily.

The second group (n=9) (Q), in which the effects of quercetin alone were investigated, received SRC, tap water to drink, and gelatine cubes containing quercetin to be delivered at a dose 100 mg/kg body weight (Sigma-Aldrich Co., St Louis, MO, USA).

The third group (n=9) (F) received SRC, a 20% fructose solution (Li et al., 2016), and plain gelatine cubes daily to induce NAFLD and NAFPD.

A fourth group (n=9) (F + Q), in which the prophylactic effects of quercetin against high fructose induced NAFLD and NAFPD were investigated, received SRC, 20% fructose solution, and gelatine cubes containing quercetin to be delivered at a dose of 100 mg/kg body weight daily.

The treatment period lasted for 12 weeks, which was based on previous studies that successfully induced metabolic dysfunction using dietary fructose in rats within 8 weeks (Mihajlova et al., 2017) and 12 weeks (Elshazly et al., 2020).

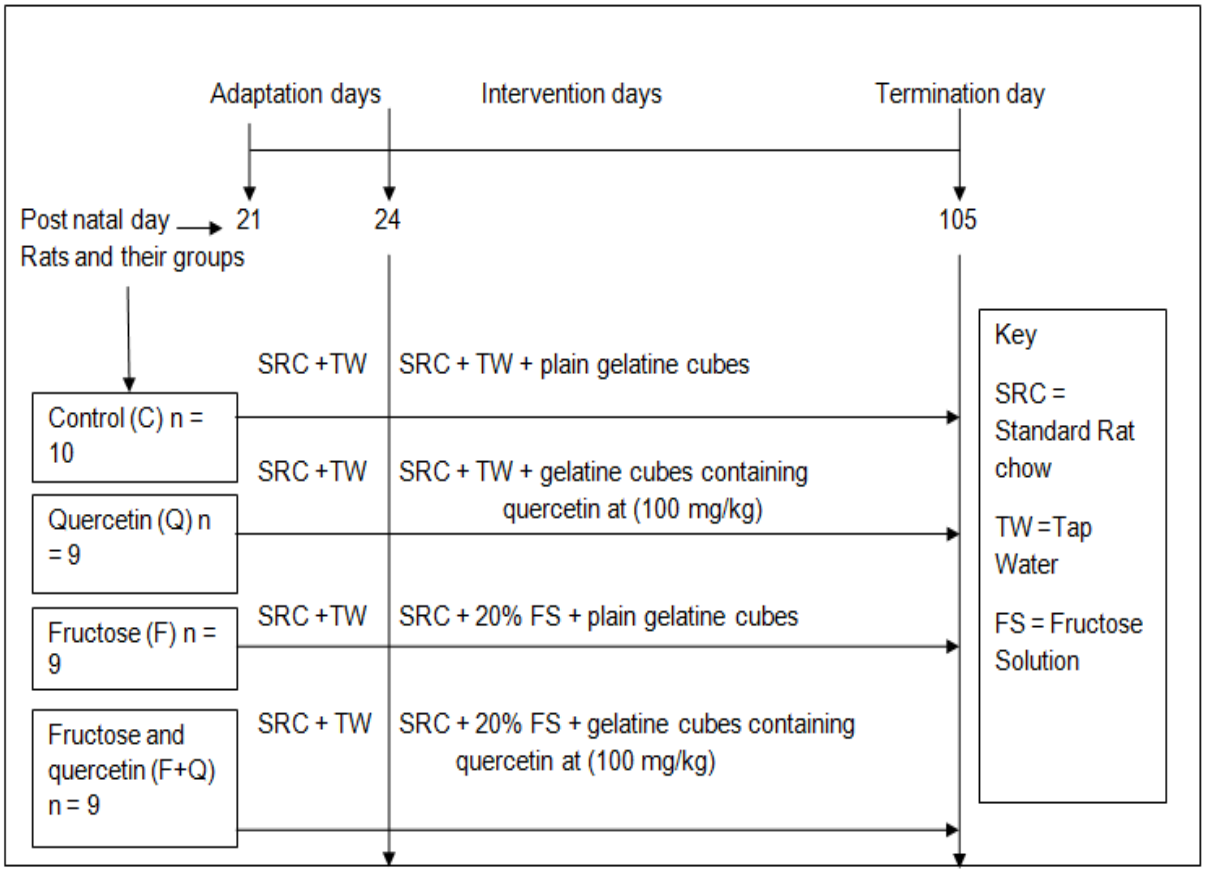

Figure 2.1 Diagram illustrating study design, experimental groups, and the timeline for the study investigating whether the flavonoid quercetin prevents the development of non-alcoholic fatty liver disease and non-alcoholic fatty pancreatic disease in growing female Sprague Dawley rats fed a high fructose diet for 12 weeks.

2.4. Feeding

The rats had *ad libitum* access to their respective diets and 20% fructose solution or tap water, throughout the 12 week study period. The commercially manufactured standard rat chow (SRC) used in this study was procured from LabChefresearch nutrition (LabChef, North West University/RB2005); see nutritional value (Table 2.1). Two weeks prior to termination, food (rat chow), tap water, and 20% fructose solution intake were measured weekly, in order to track the group with rapid growth and subsequently development of metabolic dysfunctions. At the end of the 12 weeks, rats were fasted overnight before termination.

Table 2.1 Nutritional composition of the commercially manufactured standard rat chow

Ingredients	**Quantity**
Crude protein	200 g/kg
Crude fats and oil	50 g/kg
Crude fibre	40 g/kg
Crude ash	70 g/kg
Calcium	12 g/kg
Phosphorus	7.5 g/kg
Linoleic acid	12 g/kg
Vitamin A	16000 IU/kg
Vitamin D	2000 IU/kg
Vitamin E	100 mg/kg

2.5. Fructose Solution and Treatment Preparation

The twenty percent (20%) fructose solution was made based on weight by volume formula, in which 200 g of fructose (Huletts®, Fructose Concentrated Sweetness, Low GI, South Africa) was diluted in one litre of tap water (Mamikutty et al., 2014, Mamikutty et al., 2015). Two drops of red or green food colourant (Robertsons Food Colouring, Libstar Operations (Pty) Ltd, South Africa) were added to the stock solutions and tap water, respectively, for ease of recognition of the different fluids. Gelatine cubes (2 ml volume) were made using 8 g gelatine (Davis gelatine, Johannesburg, South Africa), 8 g brown sugar (Tongaat-Hulett South Africa Ltd), 4ml Bovril (Bovril, Unilever, Johannesburg, South Africa) in 100 ml of distilled warm water at 50°C. The method used to make the gelatine cubes, a modified version from Kamerman et al. (2004), is described by Donaldson et al. (2019). The solution with

or without quercetin was then transferred into ice tray moulds and allowed to set in a fridge. The gelatine cubes (2 ml volume) were constituted and used as a vehicle to administer quercetin at a dose of 100 mg/kg body weight daily. The chosen dose of quercetin has been used in a previous metabolic study (Kim et al., 2011).

2.6. Body Mass Measurements, Food, Fluid, and Total Caloric Intake

2.6.1. Body Mass measurements

All rats were weighed twice a week, using an electronic digital scale (Precisa, 310M, Precisa Instruments, Switzerland) throughout the experimental period. The body weights were used to monitor the rats' general wellbeing and to enable us to adjust the quercetin concentration appropriately, to ensure delivery of a consistent dosage according to body weight.

2.6.2. Food, Fluid and total caloric intake

The food and fluid consumption by each rat was determined weekly in the last 2 weeks i.e. 11^{th} and 12^{th} weeks of the experiment. At the beginning of each week a known amount of food in grams (g) and fluids in millilitres (ml) were placed in each individual rat's cage. At the end of the week the amount of the food and fluid remaining were calculated and subtracted from the total amounts supplied at the beginning of the week. The weekly food and fluid consumption were computed as a percentage of body mass as g/100 g and ml/100 g, respectively. The total weekly calorie intake was calculated by multiplying the amount of food and fructose consumed each week by their respective reference calorie values, and the two were then added together.

2.7. Terminal Procedures

At the end of the intervention period (on postnatal day 104), the rats were fasted overnight with free access only to plain drinking water. The rats were then euthanized by administering an overdose of sodium pentobarbitone (Euthapent, Kyron Laboratories, Johannesburg, South Africa) at 200mg/kg body mass intraperitoneally, by a veterinarian registered with the South African Veterinary Council (Prof Kennedy H. Erlwanger). Prior to euthanasia, a few drops of blood were obtained from the tail vein of each rat using a needle prick. The blood was used to

measure fasting blood glucose levels using a calibrated glucose meter (Ascensia, EliteTM Blood glucose meter, Bayer Corporation, Mishawaka, USA), as well as haemoglobin (Hb) and packed cell volume (PCV) using an HCt (Haematocrit) meter (Afrimedics, Highlands North, Johannesburg, South Africa).

2.8. Collection of Blood and Tissues

Following euthanasia, the thoracic cavity was opened and a cardiac puncture was performed, using a 21G needle and 10ml syringe for blood collection. The blood was collected into both plain and lithium heparinised tubes (Kendon Medical Supplies (Pty) Ltd, Gemini Street, LinbroBusiness Park Sandston, Johannesburg, South Africa) for serum and plasma collection respectively. The liver, pancreas, and intra-abdominal visceral fat were dissected out and their masses were weighed using an electronic balance (Precisa, 310M, Precisa Instruments, Switzerland). A segment of the liver from the right medial lobe and the pancreas were collected and immersed into 10% phosphate buffered formalin for fixation and storage until further analysis. The rest of the liver was collected and placed individually in plastic bags which were then sealed and stored at -20˚C for further analysis.

2.9. Processing and Storage of Plasma and Serum

The blood samples collected upon termination were centrifuged (SorvalIIRT®6000B, DuPont Instruments, New York, USA) at 5000 x g per minute for 20 minutes at room temperature. The plasma and serum were then collected into micro tubes (Eppendorf, Hamburg, Germany) and stored at 80˚Cin a freezer (Esco Lexicon® II ULT freezerAdelab scientific, Holland Street, Thebarton, South Australia) until further biochemical analysis.

2.10. Biochemical Health Profile Markers

An hour before the assays were performed, the stored frozen serum was brought to room temperature. Serum concentrations of alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, albumin, total protein, amylase, lipase, and total bilirubin were determined using an IDEXX VetTest Chemistry Analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per manufacturer's instructions. The Mean Corpuscular Haemoglobin Concentration (MCHC) was computed using the formula (Wakeel et al., 2020):

$$\text{MCHC in (g/dl)} = \text{Hb(g/dl)} / \text{PCV(\%)} \times 10$$

2.11. Serum Insulin Concentration and Computation of the Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR)

Fasting serum insulin concentrations were determined using a rat specific Insulin Enzyme Linked Immuno Sorbent Assay (ELISA) kit (Elabscience Biotechnology Co., Ltd), in accordance with the manufacturer's instructions (Appendix C). This (ELISA) kit has detection range of 0.31 to 20ng/ml. The insulin resistance index was then computed according to the Homeostasis Model of Assessment (HOMA-IR) using the following formula below (Cacho et al., 2008, Belobrajdic et al., 2012):

$$\text{HOMA} - \text{IR} = [\text{FPG(mg/dl)} \times \text{FPI(µU/ml)}] \div 2.43$$

FPG = Fasting Plasma Glucose, FPI = Fasting Plasma Insulin

2.12. Serum Adiponectin Concentration

Fasting serum adiponectin concentrations were determined using a rat specific adiponectin (ADP/Acrp30) Enzyme Linked Immuno Sorbent Assay (ELISA) kit (Elabscience Biotechnology Co., Ltd), in accordance with the manufacturer's instructions (Appendix D). This (ELISA) kit has detection range of 46.88 to 3000pg/ml.

2.13. Total Cholesterol (TC), High-Density Lipoprotein Cholesterol (HDL-C), and Low-Density Lipoprotein Cholesterol (LDL-C) Assays

A colorimetric assay kit (single reagent, COD-PAP method) (Elabscience Biotechnology Co., Ltd), with detection range of 0.09 to 25.85 mmol/l was used for determining the total cholesterol concentration, according to the manufacturer's instructions (Appendix E). The optical densities of the blank, standard, and samples were measured. Ten (10) µL of each sample, standard, and blank were added to separate test tubes, followed by the addition of 1000 µL of enzyme working solution (made of cholesterol esterase, cholesterol oxidase, peroxidase, phenol, and Good's buffer) to all the test tubes before incubation for 10 minutes at 37˚C.Finally the optical densities (OD) of all the samples, standard and blank were measured using a spectrophotometer (Beckman Coulter, South Africa, Pty Ltd) at 510 nm using 0.5 cm diameter cuvette. The 10minute incubation period was to allow for complete

enzymatic reactions between the enzyme working solution and the samples to occur. Following the enzymatic reaction, a red colour quinone compound is formed, which is directly proportional to the cholesterol content of a given sample. The average optical density of the blank and standards was taken before the TC concentrations (Conc) were calculated using the formula:

$$\text{TC Conc} = [(\text{OD Sample} - \text{OD Blank}) \div (\text{OD Standard} - \text{OD Blank})] \times \text{Conc of standard } (5.17\ \text{mmol/l})$$

A Low density lipoprotein cholesterol (LDL-C) colorimetric assay kit (Double reagents) and a High density lipoprotein cholesterol (HDL-C) colorimetric assay kit (Double reagents) (Elabscience Biotechnology Co., Ltd) were used to determine serum LDL-C and HDL-C concentrations respectively, following the manufacturer's instructions (Appendix F and Appendix G). These ELISA kits used for LDL-C and HDL-C have detection ranges of (0.2 to 12 mmol/l) and (0.065 to 3.8 mmol/l) respectively.

The optical density of the blanks, standards, and sample's LDL-C and HDL-C were measured in duplicate. The first optical densities (A1Samples, A1Blanks, and A1Standard) were measured, after 10 µL of the blank, samples and standards were pipetted in to separate test tubes. Before adding 750 µL of reagent 1 (made of Good's buffer, Toos, $MgCl_2 \cdot 6H_2O$, cholesterol oxidase, and peroxidase) to all the test tubes and incubated for 5 minutes at 37˚C.The second optical densities (A2Samples, A2Blank, and A2Standard) were recorded, following the addition of reagent 2 (made of Good's buffer 4-ampyrone, $MgCl_2 \cdot 6H_2O$, cholesterol esterase, and surfactant), the mixture was then incubated for 5 minutes at 37˚C. The five minute incubation period was to allow for complete chemical reactions to occur in the mixture, which ends with the formation of a red/purple pigment. The red/purple pigment is directly proportional to the lipid content of the samples; the first optical densities were measured with a spectrophotometer (Beckman Coulter, South Africa, Pty Ltd) at a wavelength of 546 nm. The HDL-C and LDL-C concentrations of each sample were calculated using the respective formulas below:

$$\text{HDL} - \text{C} = [(\text{A2Sample} - \text{A1Sample}) - (\text{A2Blank} - \text{A1Blank})] \div [(\text{A2Standard} - \text{A1Standard}) - (\text{A2Blank} - \text{A1Blank})] \times \text{Conc of Standard } (1\ \text{mmol/l})$$

$$\text{LDL} - \text{C} = [(\text{A2Sample} - \text{A1Sample}) - (\text{A2Blank} - \text{A1Blank})] \div [(\text{A2Standard} - \text{A1Standard}) - (\text{A2Blank} - \text{A1Blank})] \times \text{Conc of Standard } (2.4\text{mmol/l})$$

2.14. Hepatic Lipid Yield Determination

The Soxhlet method (AOAC Official Method 920.39) was used for hepatic lipid extraction. The liver samples from individual rats were lyophilised using a freeze dryer (Model BK-FD12, SP Scientific, New York, USA) and then homogenised to form fine particles (Appendix H). Each homogenised liver sample (0.5 g) was placed on cotton wool, soaked with petroleum ether in the extraction thimble and 200 ml of petroleum ether was put into the pre weighed round flask on the heating pad, set at 50˚C.

The flask was connected to the extraction chamber and extraction was run for 2 hours. Thereafter, the round bottom flasks with the petroleum ether and oil were evaporated to dryness under vacuum, using a rotor evaporator (Labocon (Pty) Ltd, Krugersdorp, South Africa). The round bottom flask containing the extracted oil from the liver sample was allowed to cool before it was weighed and the percentage lipid content of the liver samples was then calculated as:

$$\%\ \text{liver lipids} = [\text{weight of the flask with extracted oil} - \text{weight of the empty flask}] \div \text{Mass of the sample}] \times 100$$

2.15. Hepatic and Pancreatic Histomorphology

2.15.1. Hepatosomatic and pancreatosomatic indices

The Hepatosomatic and pancreatosomatic indices were determined by dividing the total weight of each tissue by the total terminal body weight and then expressing it as a percentage, as detailed in the formulae (Zeng et al., 2012, Jan and Ahmed, 2016).

$$\text{HSI}\ (\%) = [\text{Liver Wet Weight}\ (\text{g}) \div \text{Total Body Weight}\ (\text{g})] \times 100$$

$$\text{PSI}\ (\%) = [\text{Pancreatic Wet Weight}\ (\text{g}) \div \text{Total Body Weight}\ (\text{g})] \times 100$$

HSI= Hepatosomatic index, PSI=pancreatosomatic index

2.15.2. Histology of the liver and pancreas

The already fixed liver segments from the right medial lobe and half of the pancreatic gland were processed separately using an automated tissue processing machine (Thermo Scientific™ Citadel 2000 Tissue processor, Thermo Fischer Scientific, USA) for 24 hours. The tissues were then embedded (block out) within paraffin wax and sectioned using a microtome (Leica Biosystems, USA), set at 5 µm thicknesses. Two sections of the tissue were placed on a glass slide. One slide each for liver and

pancreas were stained with Masson’s trichrome (MT) and haematoxylin and eosin (HE) in accordance with the standard protocols (Bancroft et al., 2018) for the assessment of fibrosis and steatosis/inflammation, respectively. MT is a three dyes staining protocol used to examine histological samples for fibrosis in tissues including the liver and pancreas. The procedure was done as follows; slides were deparaffinised and rehydrated with 95% alcohol, rinsed under running tap water for 10 minutes, stained with Weigert's working hematoxylin for 10 minutes. Before, rinsing in running tap water for 5 minutes and subsequently washed in distilled water. Slides were then stained with Biebrich scarlet acid fuchsin for 10 minutes, rinsed in distilled, differentiated in Phosphotungstic-phosphomolybdic acid for 10 minutes, transferred directly into Aniline blue for 5 minutes and briefly rinsed in distilled water. The slides were then differentiated in 1% Acetic acid for 2 minutes, washed in distilled water, dehydrated through 95% ethyl alcohol and clear in xylene and finally cover slips were mounted.

On the other hand HE stain was carried out as follows; sections were dewaxed through xylene, 100% alcohol, 95% alcohol and water, before staining in hematoxylin for 3 minutes. Sections were then washed in running tap water for 5 minutes, differentiated in 1% acid alcohol for 5 to 10 seconds, washed in tap water for 10 to 15 minutes, and stained with eosin for 10 minutes. Finally, the sections were dehydrated through 95% alcohol and 100% alcohol and cleared in xylene, before mounting the cover slips. Following staining, three (3) fields were viewed per slide with a Leica ICC59 HD video camera, connected to a Leica DM 500 microscope. The photomicrographs of the stained sections were then analyzed and histological features of both the liver and pancreas sections were semi quantitatively and quantitatively assessed while blinded to the animal treatment.

2.15.3. Hepatic steatosis and inflammation scoring procedure

Liver sections stained with haematoxylin and eosin were assessed for steatosis, inflammation and hepatocellular ballooning according to the non-alcoholic fatty liver disease scoring system, described by Brunt et al. (2011). Hepatic steatosis was determined by analyzing the hepatocellular vesicular steatosis, based on the total area affected and grading was done (Table 2.2). The microsteatosis was defined as the lipid droplet within the hepatocyte without affecting the nucleus position, while

macrosteatosis was defined as the lipid droplet that scattered the hepatocyte or occupied most of the hepatocyte pushing the nucleus to the periphery. Inflammation and hepatocellular ballooning were determined by counting the number of inflammatory cell aggregates in the liver parenchyma and grading was done (Table 2.2). The components of NAFLD Activity Score (NAS) are hepatic steatosis which range from 0 to 3 scores, lobular inflammation which ranges from 0 to 3, and hepatocyte ballooning which ranges from 0 to 2 (Table 2.2). The total scores of NAS are used to classify the NAFLD into 3 types (i) non-alcoholic steatohepatitis (NASH), (ii) not NASH and (iii) borderline or possible NASH based on the sum of the component scores (Table 2.4).

Table 2.2 Hepatic steatosis/lobular inflammation/hepatocellular ballooning scoring system (Brunt et al., 2011)

NAS Scores	Hepatic steatosis	Lobular inflammation	Hepatocellular ballooning
0	< 5% / Camera field of the parenchyma	No foci / camera field at × 40 magnification	No ballooned cell
1	5% – 33% / Camera field of the parenchyma	< 2 foci / camera field at × 40 magnification	Few ballooned cells
2	> 33% – 66% /Camera field of the parenchyma	2 – 4 foci / camera field at × 40 magnification	Many ballooned cells
3	> 66% / Camera field of the parenchyma	> 4 foci / camera field at × 40 magnification	

2.15.4. Hepatic fibrosis scoring procedure

The photomicrographs obtained from the liver sections stained with Masson's trichrome (MT) were quantitatively assessed for fibrosis of the portal areas at x 40 and grading was done (Santiago-Rolón et al., 2016) (Table 2.3).

Table 2.3 Hepatic fibrosis scoring system (Santiago-Rolón et al., 2016)

Fibrosis Grades	Definition/interpretation
0	Absent fibrosis
1	Perisinusoidal fibrosis (fibrosis localised to sinusoid spaces and hepatocyte only)
2	Periportal fibrosis(portal fibrosis and adjacent parenchyma)
3	Bridging fibrosis(fibrosis extending across lobules, connecting portal vein and portal tracts)
4	Cirrhosis (diffused fibrosis and parenchymal nodules)

Table 2.4 Interpretation of the NAS scores (Brunt et al., 2011)

NAS Scores	NAS Score Interpretations
> 5	Non-alcoholic steatohepatitis (NASH)
< 3	Not NASH
3 – 4	Borderline or possible

2.16. Pancreatic steatosis scoring procedure

The photomicrographs obtained from the pancreatic sections stained with haematoxylin and eosin were quantitatively assessed for steatosis at x 40 magnification (Unser et al., 2013), based on the percentage of fat cells per microscopic field according to the pancreatic lipomatosis scoring system and grading (Table 2.5) (Catanzaro et al., 2016).

The photomicrographs obtained from the pancreatic sections stained with MT were assessed for pancreatic fibrosis according to the methods described previously by (Xingjun et al., 2019). Interlobular and intralobular fibrosis were defined and assessed based on the presence of connective tissue within the interlobular and intralobular spaces, which were subsequently scored independently from zero (no fibrosis) to six (highest fibrosis), and the global scores for each slide were calculated based on the global pancreatic fibrosis score, the pancreatic fibrosis was graded as one of three possible grades (Table 2.6) (Gaujoux et al., 2010, Xingjun et al., 2019).

Table 2.5 The pancreatic lipomatosis scoring system (Catanzaro et al., 2016)

Stages	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
% of fat cells/microscopic field at 40x	0 – 7%	8 – 14%	15 – 25 %	26 – 50 %	≥ 51 %	≥ 75 %

Table 2.6 Pancreatic fibrosis scoring system (Xingjun et al., 2019)

Grades	Fibrosis	Global pancreatic scores
1	Mild	4–6
2	Moderate	7– 9
3	severe	10–12

2.17. Statistical Analysis

Data were analysed using Graph Pad Prism 7.0v for windows (Graph Pad Software, Inc., San Diego USA). All parametric data were expressed as mean ± standard deviation and analysed using a one way ANOVA, followed by Tukey post hoc tests. Nonparametric data (histological scores) were expressed as median and interquartile range and analysed using a Kruskal Wallis test, followed by a Dunn's post hoc test. Differences were considered statistically significant at a P value of < 0.05. A repeated measure analysis of variance (ANOVA) was performed for parameters measured each week (body weight, fluids intake, and food intake).

CHAPTER 3: RESULTS

3.1. Morbidity and Mortality of the Animals

There were no clinical morbidities that required any animals to be removed from the study and there were no unplanned mortalities recorded.

3.2. Effects of Orally Administered Quercetin on Growth Performance, Feed, Fluid, and Calorie Intake

3.2.1. Growth performance

3.2.1.1. *Body mass measurements*

Figure 3.1 shows the initial and terminal body masses of the rats. There were no differences (P > 0.05) in either the initial or the terminal body masses of the rats across treatment groups. At the end of the intervention, all the rats had a significant increase in body mass (P < 0.0001).

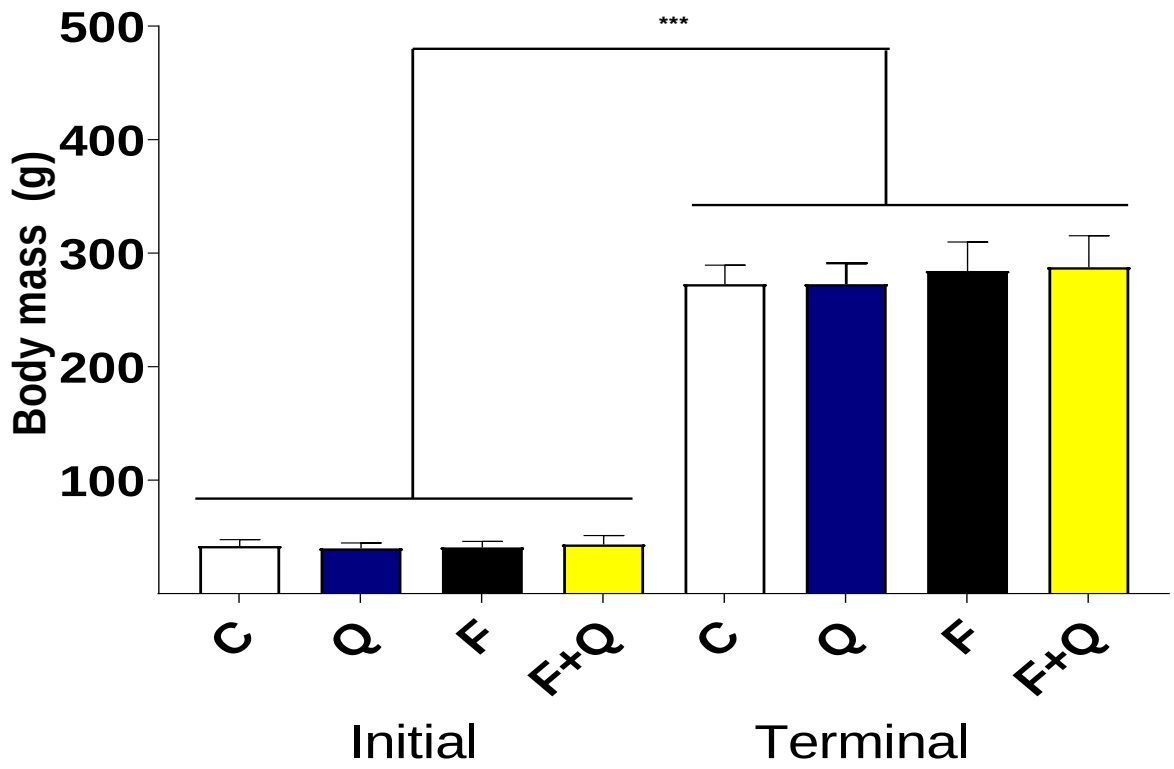

Figure 3.1 Effect of orally administered quercetin on the initial and terminal body masses of the rats.

Data was presented as mean ± standard deviation. *** P < 0.0001. C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes),

F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.2.1.2. *Percentage of body mass gain*

Figure 3.2 shows the percentage body mass gain of the rats at the end of the 12 week treatment period with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. There were no significant differences (P > 0.05) in the percentage body mass gain between treatment groups.

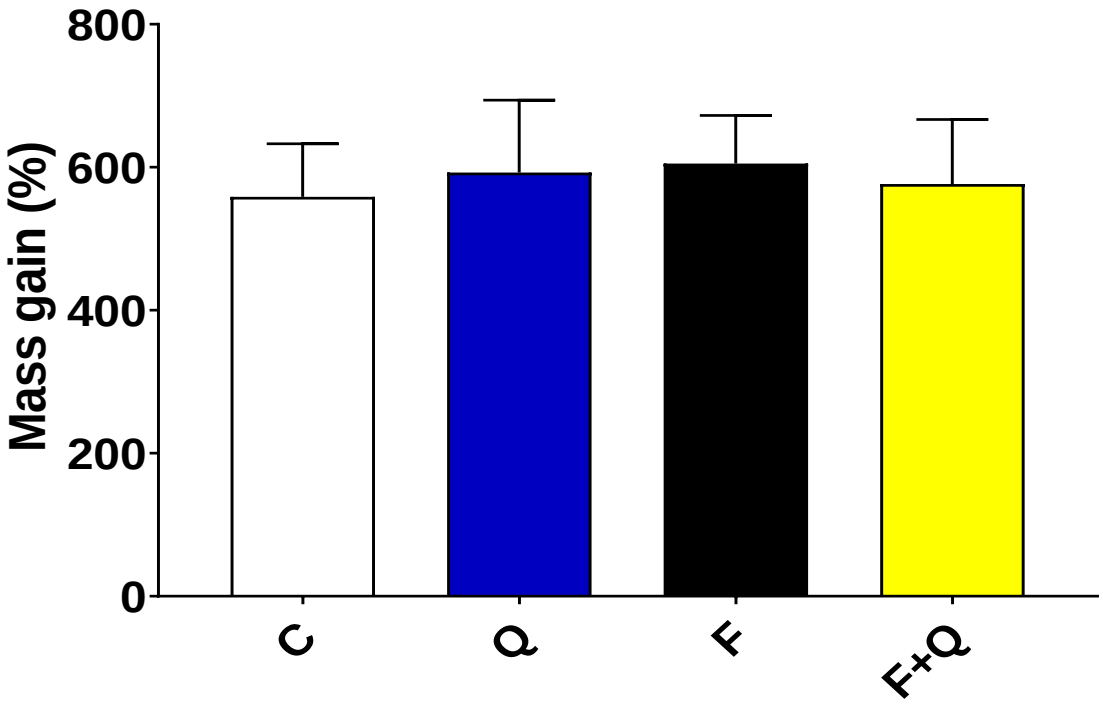

Figure 3.2 Effect of orally administered quercetin on the percentage of body mass gain of female Sprague Dawley rats.

Data was presented as mean ± standard deviation. The percentage body mass gain between treatment groups was similar (P > 0.05). C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100mg/kg in gelatine cubes). n =10 in the control group and n= 9 in the remaining groups. FS (Fructose solution), SRC (standard rat chow).

3.2.2. Feed, fluid, and calorie intake

3.2.2.1. *Feed intake*

Table 3.1 shows feed (SRC) intake of the rats during the 11th and 12th week of the treatment period with either a standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. The rats in the C and Q groups consumed significantly ($P < 0.05$) more feed than the rats in the F and F + Q groups, both during the 11th and the 12th weeks of the experiment.

3.2.2.2. *Fluid (water and 20% fructose solution) intake*

Table 3.1 shows the fluid intake of the rats during the final two (11th and 12th) weeks of the treatment period with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. In the 11th week of the intervention, no significant differences in fluid intake were observed ($P > 0.05$) between treatment groups. However, during the 12th week of treatment, the F + Q group consumed significantly ($P < 0.05$) more fluid than the rest of the groups.

Table 3.1 Feed intake and fluid intake of the female Sprague Dawley rats in the 11th and 12th week of the experiment

Parameters	C	Q	F	F + Q	Significance level
Week 11					
Feed intake (g/100g)	44.51 ± 3.69[a]	45.76 ± 5.25[a]	24.25 ± 4.94[b]	28.67 ± 3.36[b]	****
Feed intake (g/100g)	43.71 ± 3.39[a]	45.47 ± 41.00[a]	26.18 ± 3.01[b]	28.75 ± 3.37[b]	****
Week 12					
Fluid intake (ml/100g)	103.50 ± 10.53	104.00 ± 28.89	93.19 ± 22.93	77.30 ± 7.90	ns
Fluid intake (ml/100g)	108.00 ± 19.94[b]	97.97 ± 17.67[b]	92.57 ± 10.42[b]	85.63 ±15.33[a]	*

Data presented as mean ± standard deviation. [a, b] Different superscript letters within a row indicate significant differences at * P < 0.05, ***P<0.0001, ns (no significant difference). C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.2.2.3. *Calorie intake from feed and 20% fructose solution*

Table 3.2 shows the calorie intake (kcal/100g body weight) of the rats in the 11th and the 12th week of the treatment period with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. The total caloric intake of the rats between treatment groups during the 11th week of treatment was not different ($P > 0.05$). However, in the 12th week of the treatment, the rats in the F and F + Q groups consumed significantly ($P < 0.05$) more calories in comparison to the rats in the C and Q groups.

Table 3.2 Calorie intake of the female Sprague Dawley rats in the 11th and 12th week of the experiment

Parameters	C	Q	F	F + Q	Significance level
Calorie intake in the 11th week(kcal/100g body weight)	141.10 ± 11.71	145.10 ± 16.64	151.40 ± 19.96	152.70 ± 7.86	ns
Calorie intake in the 12th week(kcal/100g body weight)	138.50 ± 10.75^{b}	144.10 ± 13.98^{b}	157.00 ± 6.41^{a}	159.70 ± 11.20^{a}	**

Data was presented as mean ± standard deviation. $^{a, b}$ Different superscript letters within a row indicate significant differences $P < 0.05$, ns (no significant difference), C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.3. Effects of Fructose Administration on Circulating Metabolic Substrates, adiponectin, Insulin, HOMA-IR, and Visceral fat masses

3.3.1. Circulating metabolites (glucose, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentrations)

Table 3.3 shows the fasting blood glucose (FBG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) concentrations of the rats following the 12 week treatment period with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. No significant differences were observed in the FBG, TC, and LDL-C concentrations between treatment groups ($P > 0.05$, Table 3.3). However, rats in the F + Q group had significantly higher ($P < 0.05$) HDL-C concentrations compared to the rats in the control, quercetin, and fructose groups.

Table 3.3 Fasting blood glucose, total cholesterol, low density lipoprotein cholesterol, and high density lipoprotein cholesterol concentrations of female Sprague Dawley rats after 12 weeks of treatment.

Parameters	C	Q	F	F + Q	Significance level
FBG (mmol/l)	4.06 ± 0.29	4.09 ± 0.38	3.82 ± 0.23	3.89 ± 0.18	ns
TC (mmol/l)	1.95 ± 0.65	2.05 ± 0.37	2.49 ± 0.60	2.49 ± 0.57	ns
LDL-C (mmol/l)	0.13 ± 0.13	0.21 ± 0.08	0.17 ± 0.16	0.29 ± 0.14	ns
HDL-C (mmol/l)	4.25 ± 0.83^{b}	4.57 ± 0.72^{b}	4.51 ± 0.45^{b}	5.20 ± 0.80^{a}	*

Data presented as mean ± standard deviation. ^{a}, ^{b}, Different superscript letters within a row indicate significant differences * $P < 0.05$, ns (no significant difference). C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups, FS (Fructose solution), SRC (Standard rat chow), FBG (fasting blood glucose), TC (Total cholesterol), LDL-C (low density lipoprotein cholesterol), HDL-C (High density lipoprotein cholesterol).

3.3.2. Serum insulin, adiponectin and homeostatic model assessment of insulin resistance (HOMA-IR) index

Table 3.4 shows the serum insulin concentrations, HOMA-IR indices, and adiponectin concentrations of the rats following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. Serum insulin and adiponectin concentrations, as well as the computed HOMA-IR indices, were not significantly different between treatment groups ($P > 0.05$, Table 3.4).

Table 3.4 Serum insulin concentrations, HOMA-IR indices, and serum adiponectin concentrations of female Sprague Dawley rats after 12 weeks of treatment.

Parameters	**C**	**Q**	**F**	**F + Q**	**Significance level**
Insulin (ng/ml)	1.20 ± 0.44	1.19 ± 0.49	1.19 ± 0.55	1.50 ± 0.53	ns
HOMA-IR Index	0.72 ± 0.25	0.72 ± 0.29	0.67 ± 0.31	0.87 ± 0.30	ns
Adiponectin (pg/ml)	424.20 ± 187.50	344.80 ± 203.60	343.60 ± 215.40	383.40 ± 288.10	ns

Data presented as mean ± standard deviation. ns (no significant difference). C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow), HOMA-IR= homeostatic model assessment of insulin resistance index.

3.3.3. Visceral fat masses

Figure 3.3 shows the visceral fat masses of the rats following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. The visceral fat mass was significantly higher in the F group compared to the control group ($P < 0.05$, Figure 3.3). The visceral fat mass was significantly higher in the F + Q group compared to the Q group ($P = 0.0080$). There were no differences in the visceral fat mass between the F and F + Q groups and the C, Q groups ($P > 0.05$).

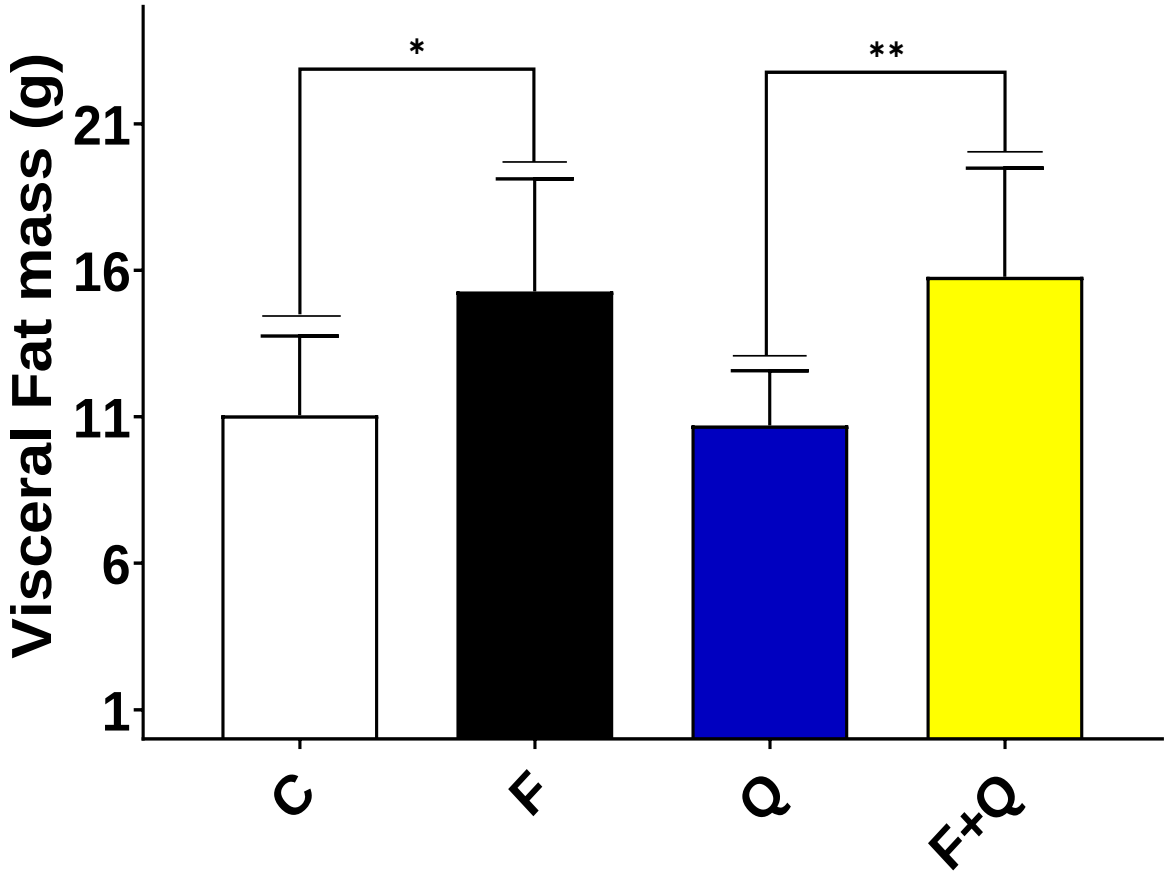

Figure 3.3 Effect of orally administered quercetin on the visceral fat masses of female Sprague Dawley rats following 12 weeks of treatment.

Data presented as mean ± standard deviation.* $P < 0.05$, ** $P < 0.0001$. C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20%

FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.4. Red Blood Cell Indices

Table 3.5 shows erythrocyte indices of the rats after 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. No significant differences were observed (P > 0.05, Table 3.5) in any of the general health markers across treatment groups.

Table 3.5 Erythrocyte indices of the female Sprague Dawley rats after 12 weeks of treatment.

Parameters	C	Q	F	F + Q	Significance level
PCV (%)	50.70 ± 2.63	50.00 ± 2.92	50.56 ± 1.59	49.56 ± 3.17	ns
Hb (g/dl)	16.85 ± 0.83	16.62 ± 0.97	16.87 ± 0.57	16.50 ± 1.08	ns
MCHC (g/dl)	3.32 ± 0.02	3.32 ± 0.02	3.34 ± 0.02	3.33 ± 0.14	ns

Data presented as mean ± standard deviation. ns (no significant difference), C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow), PCV (packed cell volume), Hb (Haemoglobin), MCHC (mean corpuscular haemoglobin concentration).

3.5. Markers of Renal, Hepatic and Pancreatic Function

3.5.1. Markers of renal function

Table 3.6 shows markers of renal function of the rats after 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. The serum blood urea nitrogen, creatinine, and urea to creatinine ratio were not significantly different between treatment groups (P > 0.05, Table 3.6).

Table 3.6 Markers of renal function of the female Sprague Dawley rats after 12 weeks of treatment

Parameters	C	Q	F	F + Q	Significance level
BUN (mg/dl)	18.90 ± 1.85	20.33 ± 2.00	19.00 ± 3.20	18.67 ± 3.84	ns
Creatinine (mg/dl)	0.78 ± 0.10	0.86 ± 0.27	0.84 ± 0.07	0.80 ± 0.11	ns
BUN/Creatinine ratio	24.70 ± 3.86	25.44 ± 5.98	22.56 ± 3.25	23.44 ± 3.91	ns

Data presented as mean ± standard deviation. ns (no significant difference), C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow), BUN (blood urea and nitrogen).

3.5.2. Markers of hepatic function

Table 3.7 shows markers of hepatic function of the rats after 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. The markers of liver function were similar across treatment groups ($P > 0.05$, Table 3.7).

Table 3.7 Markers of hepatic function of female Sprague Dawley rats after 12 weeks of treatment.

Parameters	C	Q	F	F + Q	Significance level
ALT (u/l)	48.20 ± 5.01	50.78 ± 7.17	43.89 ± 5.90	43.67 ± 5.22	ns
Total Bilirubin (mg/dl)	0.29 ± 0.057	0.27 ± 0.13	0.34 ± 0.14	0.30 ± 0.09	ns
Albumin (g/dl)	2.93 ± 0.22	2.97 ± 0.39	3.12 ± 0.16	3.13 ± 0.14	ns

Data was presented as mean ± standard deviation. ns (no significant difference), C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow), ALT (Alanine aminotransferases).

3.5.3. Markers of Pancreatic function

Table 3.8 shows the serum pancreatic enzyme (lipase and amylase) concentrations of the rats following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose (20% fructose solution), with or without quercetin. No significant differences ($P > 0.05$, Table 3.8) in serum lipase concentrations were observed between treatment groups. However, the serum amylase concentration was significantly ($P < 0.05$, Table 3.8) higher in the F and F + Q groups in comparison with the C and Q groups.

Table 3.8 Serum pancreatic enzyme (lipase and amylase) concentrations of female Sprague Dawley rats after 12 weeks of treatment.

Parameters	C	Q	F	F + Q	Significance level
Lipase (u/l)	165.20 ± 85.87	137.80 ± 30.14	181.70 ± 98.97	163.60 ± 65.11	ns
Amylase (u/l)	1572 ± 258.40^{b}	1508 ± 215.40^{b}	1980 ± 233.10^{a}	1917 ± 212.50^{a}	****

Data was presented as mean ± standard deviation, $^{a, b}$ Different superscript letters within a row indicate significant differences. **** $P < 0.0001$, ns (no significant difference). C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.6. Hepatic Parameters

3.6.1. Liver masses and hepatosomatic index (HSI)

Table 3.9 shows the liver masses and HSI of the rats following 12 weeks of treatment, with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose solution), with or without quercetin. The liver mass and the HSI were significantly higher in the F + Q group ($P < 0.05$, Table 3.9) in comparison to C and Q groups. However, there were no differences between the F and F + Q groups ($P > 0.05$).

Table 3.9 Liver masses and HSI of female Sprague Dawley rats following 12 weeks of treatment

Parameters	C	Q	F	F + Q	Significance level
Liver mass (g)	7.21 ± 0.67[b]	7.15 ± 0.70[b]	7.91 ± 0.65[a]	8.13 ± 0.80[a]	*
HSI (%)	2.64 ± 0.13[b]	2.62 ± 0.16[b]	2.80 ± 0.06[a]	2.83 ± 0.17[a]	*

Data was presented as mean ± standard deviation. [a], [b] Different superscript letters within a row indicate significant differences. * $P < 0.05$. C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.6.2. Hepatic lipid yield

Figure 3.4 shows the hepatic lipid yield of the rats following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose drinking fluid (20% fructose drinking fluid), with or without quercetin. The hepatic lipid yield was significantly ($P < 0.05$, Figure 3.4) lower in the C group compared with the rats in the F group. However, quercetin has decreased the hepatic lipid yield from 13.44 ± 3.44% in F group to 9.58 ± 2.73% in F + Q group.

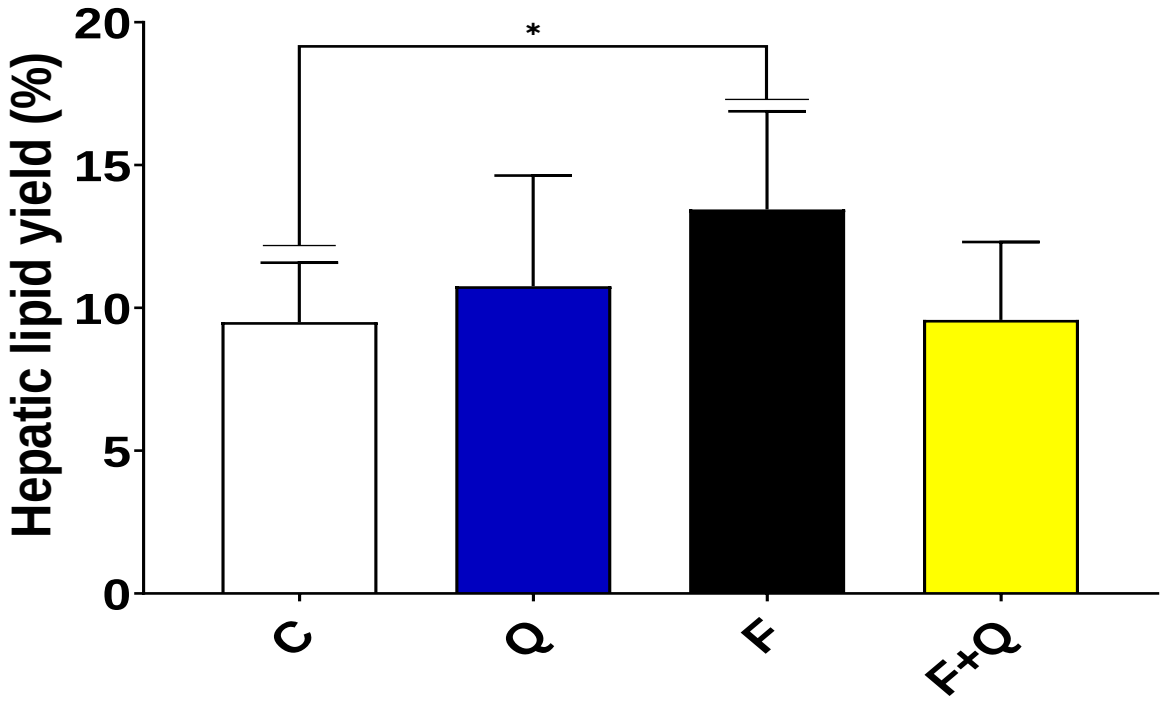

Figure 3.4 Effect of orally administered quercetin on the hepatic lipid yield of female Sprague Dawley rats following 12 weeks of treatment.

Data presented as mean ± standard deviation. * = Significantly different at $P < 0.05$. C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC +20% FS + quercetin at 100 mg/kg in gelatine cubes), n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.6.3. Hepatic macrosteatosis, microsteatosis, inflammation, and fibrosis scores

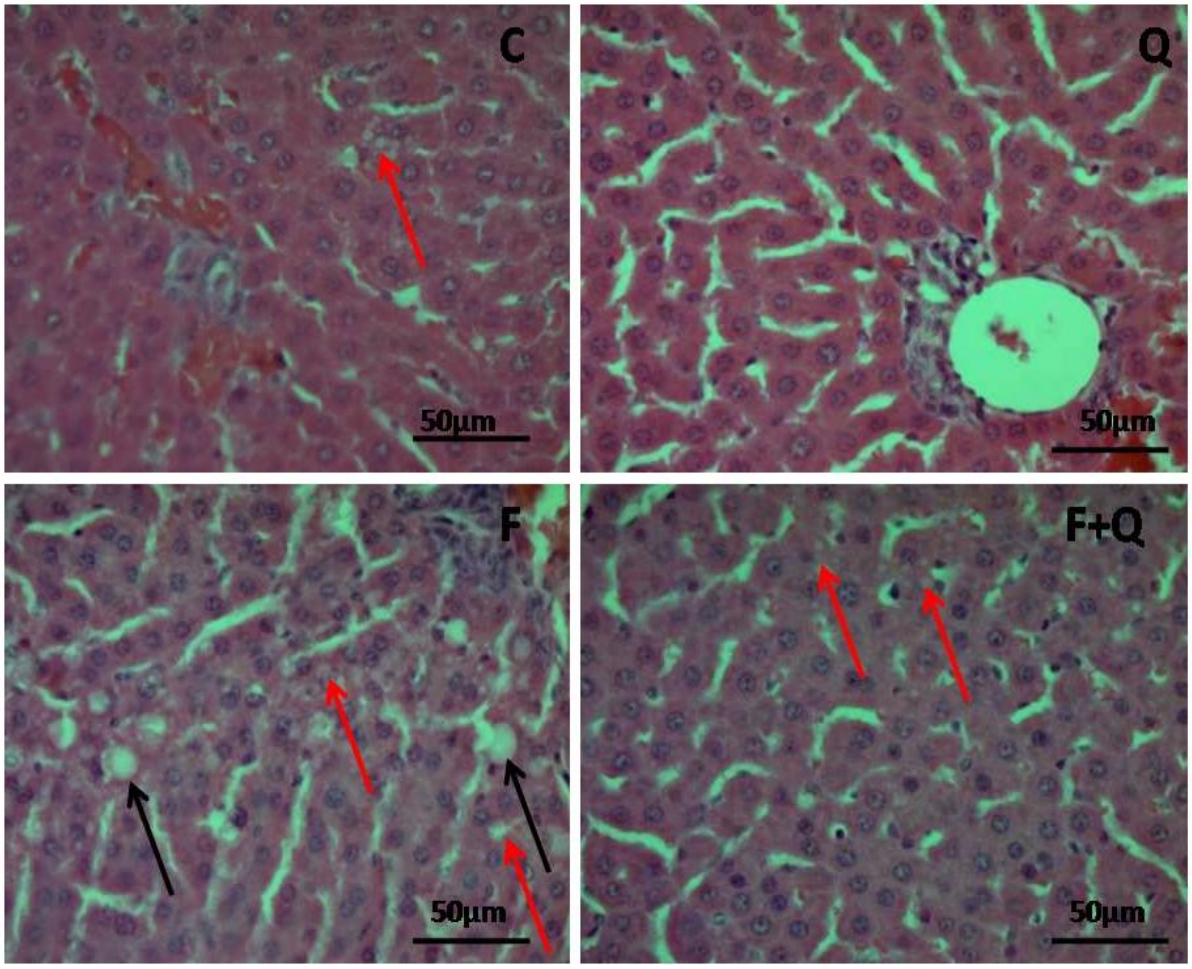

Figure 3.5 Photomicrographs showing histological features after haematoxylin and eosin staining of liver cross sections from a representative rat from each experimental treatment group. The microsteatosis is observed as the lipid droplet within the hepatocyte without affecting the nucleus position, while macrosteatosis is observed as the lipid droplet that occupied most of the hepatocyte pushing the nucleus to the periphery. Red arrows indicate microsteatosis, and black arrows indicate macrosteatosis, scale bar (50µm) in the haematoxylin and eosin stain sections.

C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes), n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

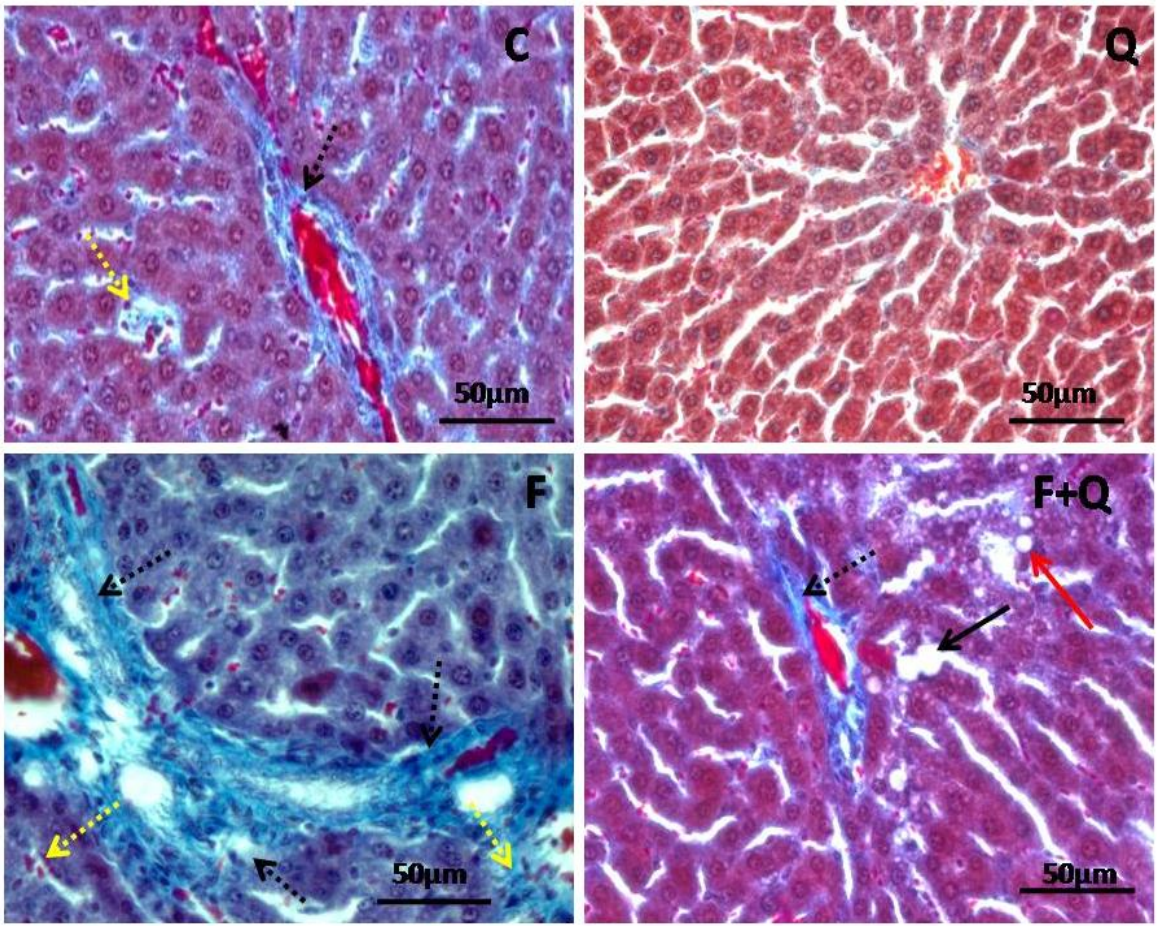

Figure 3.6 Photomicrographs showing histological features after Masson's trichrome staining of liver cross sections from a representative rat from each treatment group. The blue colour in the photomicrographs indicates the excessive deposition of fibrous tissue in the periportal and sinusoid areas, while the focus of inflammation is represented by the area of increased accumulation of neutrophils, macrophages, and the fragments from dead hepatocytes. The red arrows indicate microsteatosis, black arrows indicate macrosteatosis, dotted black arrows indicate fibrosis, and dotted yellow arrows indicate inflammatory foci, scale bar (50µm) in the MT stain sections.

C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

Table 3.10 shows the hepatic macrosteatosis, hepatic microsteatosis, hepatic inflammation, and hepatic fibrosis scores of the rats following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose (20% fructose solution), with or without quercetin.

The macrosteatosis was significantly ($P < 0.05$) higher in the F group compared to C, Q, and F + Q groups (Table 3.10 and Figure 3.5). Microsteatosis (Figure 3.5), inflammation, and fibrosis were all significantly ($P < 0.05$) higher in the F group compared to the Q and F + Q groups (Table 3.10 and Figure 3.6).

Table 3.10 Hepatic macrosteatosis, microsteatosis, inflammation, and fibrosis scores of female Sprague Dawley rats after 12 weeks of treatment.

Parameters	C	Q	F	F + Q	Significance level
Macrosteatosis	0.13 ± 0.28^{a}	0.00 ± 0.00^{a}	0.85 ± 0.96^{b}	0.04 ± 0.11^{a}	**
Microsteatosis	1.52 ± 0.94	0.74 ± 0.58^{a}	2.34 ± 0.71^{b}	0.87 ± 0.54^{a}	**
Inflammation	0.33 ± 0.35	0.11 ± 0.24^{a}	0.74 ± 0.32^{b}	0.15 ± 0.17^{a}	**
Fibrosis	0.60 ± 0.41	0.11 ± 0.17^{a}	0.93 ± 0.32^{b}	0.14 ± 0.17^{a}	***

Data presented as mean ± standard deviation $^{a, b}$ Different superscript letters within a row indicate significant differences. ** $P < 0.01$, *** $P < 0.0001$. C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.7. Pancreatic Parameters

3.7.1. Pancreatic masses and pancreatosomatic index (PSI)

Table 3.11 shows the pancreatic masses and PSI of the rats following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose (20% fructose solution), with or without quercetin. The pancreas mass and

pancreatosomatic index measured between treatments groups was not significantly different (P > 0.05, Table 3.11).

Table 3.11 Pancreatic masses and pancreatosomatic index (%) of female Sprague Dawley rats after 12 weeks of treatment

Parameters	C	Q	F	F + Q	Significance level
Pancreas mass (g)	1.04 ± 0.22	1.06 ± 0.28	1.05 ± 0.20	1.11 ± 0.11	ns
PSI (%)	0.38 ± 0.08	0.35 ± 0.10	0.37 ± 0.08	0.39 ± 0.06	ns

Data presented as mean ± standard deviation, ns (no significant difference), C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow).

3.7.2. Pancreatic steatosis

Figure 3.7 Photomicrographs showing histological features after haematoxylin and eosin staining of pancreas cross sections from a representative rat from each treatment group following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose (20% fructose solution), with or without quercetin. There were no differences between the treatment groups (Figure 3.7).

3.7.3. Pancreatic fibrosis

Figure 3.8 Photomicrographs showing histological features after Masson's trichrome staining of pancreas cross sections from a representative rat from each treatment group, following 12 weeks of treatment with either standard rat chow, with or without quercetin, or a high fructose (20% fructose solution), with or without quercetin. Therefore, in this current study the expected 20% fructose solution induced metabolic dysfunction (pancreatic fibrosis) (Figure 3.8) in the pancreas was not observed hence the prophylactic effect of quercetin against NAFPD couldn't be evaluated.

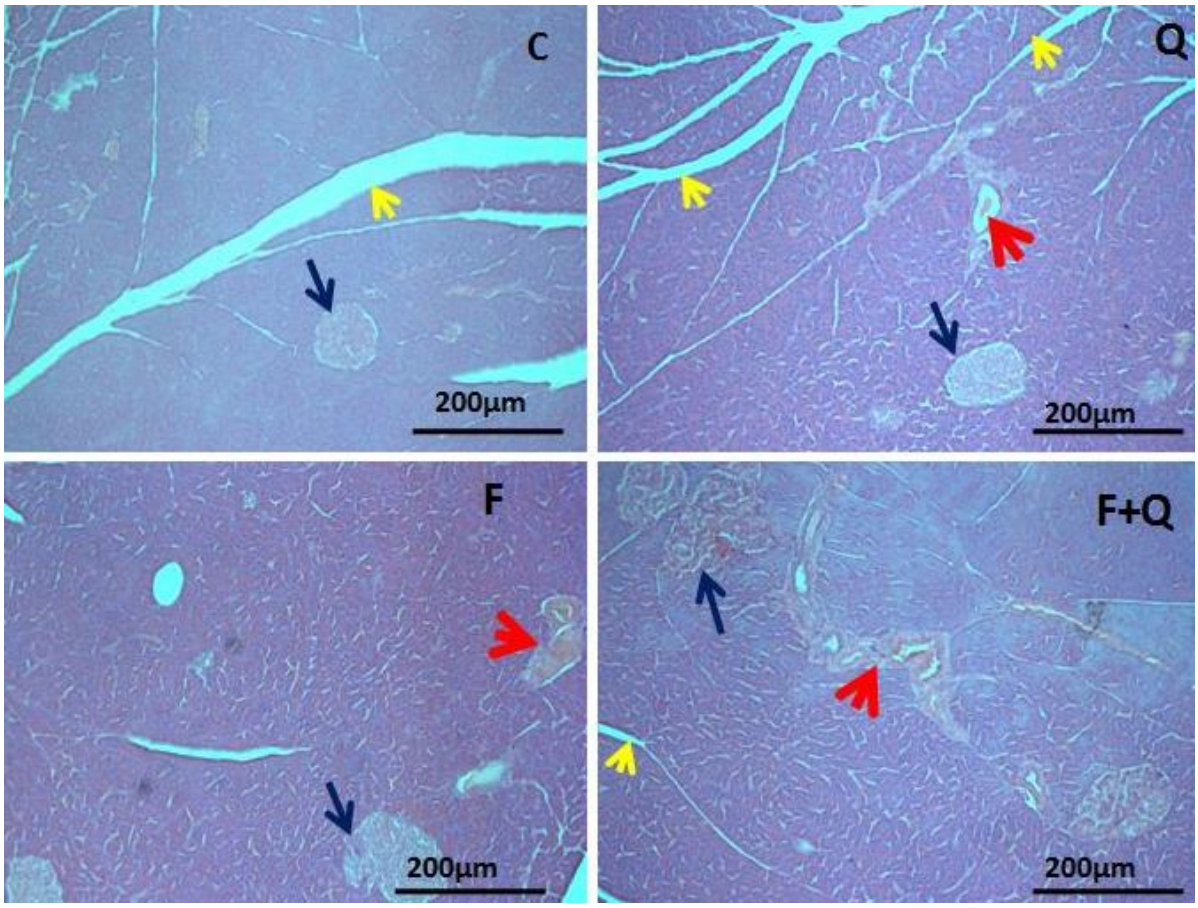

Figure 3.7 Photomicrographs showing histological features after haematoxylin and eosin staining of pancreas cross sections from a representative rat from each treatment group.

C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups, FS (Fructose solution), SRC (Standard rat chow), red arrows indicate blood vessel, blue arrows indicate (pancreatic islets) or islets of Langerhans, yellow arrows indicate interlobular spaces, scale bar (200µm) in the haematoxylin and eosin stain sections.

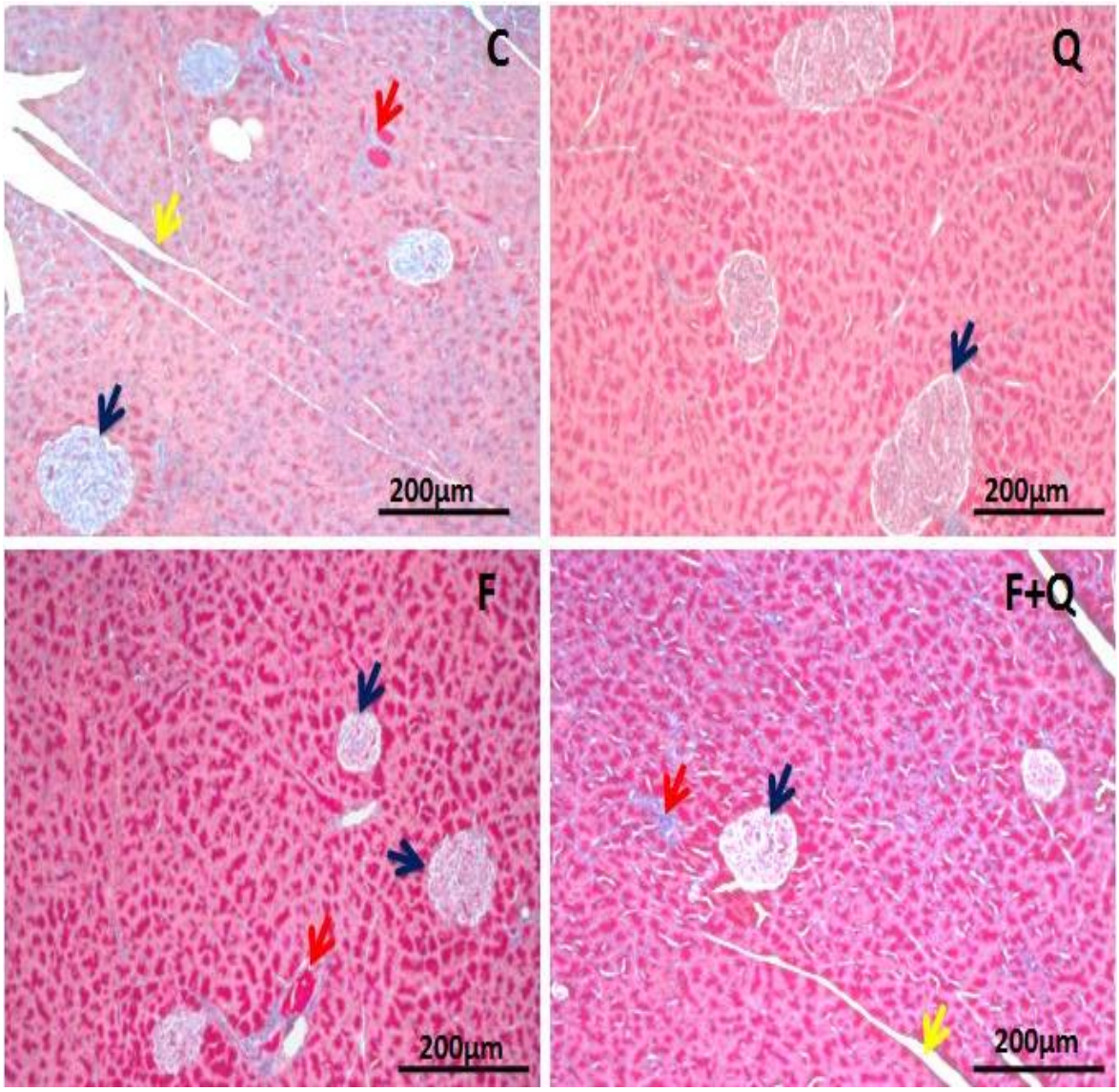

Figure 3.8 Photomicrographs showing histological features after Masson's trichrome staining of pancreas cross-sections from a representative rat from each treatment group.

C (control group) = (SRC + tap water + plain gelatine cubes), Q (quercetin group) = (SRC + tap water + quercetin at 100 mg/kg in gelatine cubes), F (fructose group) = (SRC + 20% FS + plain gelatine cubes), F + Q (fructose + quercetin group) = (SRC + 20% FS + quercetin at 100 mg/kg in gelatine cubes). n = 10 in the control group and n = 9 in the remaining groups. FS (Fructose solution), SRC (Standard rat chow), red arrows indicate blood vessel, blue arrows indicate (pancreatic islets) or islets of Langerhans, yellow arrows indicate interlobular spaces, scale bar (200µm) in the MT stain sections.

CHAPTER 4: DISCUSSION

4.1. Overview

The current study was carried out on growing, female, Sprague Dawley rats to investigate the prophylactic effects of orally administered quercetin against the metabolic dysfunction (NAFLD and NAFPD) induced by ingestion of a high fructose diet (20% fructose solution). At the end of the 12 weeks of high fructose feeding (20% fructose solution) *ad libitum*, there were no differences in terminal body mass and percentage body mass gain between treatment groups. However, all the rats grew significantly over the treatment period.

The fructose consumption significantly reduced the food intake and increased the overall calorie consumption of the rats. Although, the fructose administration increased visceral fat mass of the rats, it had no significant effect on the markers of insulin resistance (serum insulin, adiponectin, homeostatic model assessment of insulin resistance (HOMA-IR) index) and circulating metabolites. However, the liver mass, hepatosomatic index, and hepatic lipid yield were increased by fructose consumption. Furthermore, fructose consumption induced NAFLD as evidenced by the significant hepatic macro and microsteatosis, as well as inflammation and fibrosis present in the livers of rats fed the fructose solution. The hepatic macro and microsteatosis, as well as the inflammation and fibrosis were all significantly reduced by the quercetin treatment. Despite the changes in visceral fat mass, liver mass, hepatosomatic index, hepatic lipid yield and the presence of NAFLD. Neither the fructose treatment nor the quercetin administration had any significant effect on the red blood cell indices, markers of hepatic and renal function or the circulating metabolites, except for the HDL-C level which was increased by quercetin treatment. Similarly, both the fructose treatment and the quercetin administration had no significant effects on pancreatic mass, pancreatosomatic index, pancreatic histopathology and plasma lipase concentration. Additionally, the quercetin treatment failed to reduce the plasma amylase level that was increased by consumption of the 20% fructose solution. Subsequently, these findings will be discussed in detail.

4.2. Growth Performance, Food, Fluid, and Total Calorie Intake

High fructose diets have been linked with excessive increases in body mass and the development of obesity, secondary to excessive calorie consumption from the high fructose diet (Cox et al., 2012, Mamikutty et al., 2014, Elshazly et al., 2020). In this study, the induction body masses of all the rats within the different treatment groups

were similar and all the rats grew significantly during the study period. At the end of the 12 weeks treatment, neither the 20% fructose solution, nor the quercetin supplementation significantly altered the body mass and percentage body mass gain of the growing female rats. Similarly, a previous study also observed no significant differences in terminal body mass or body mass gain between treatment groups at the end of 12 weeks of feeding a 20% fructose solution to 21 days old female Sprague Dawley rats (Gumede et al., 2020b). Muhammad et al. (2019), also fed 21 days old female Sprague Dawley rats a high fructose diet (20% fructose solution) for 10 weeks and observed no significant difference in terminal body mass between treatment groups.

Even though body mass was not different between treatment groups in the current study, total calorie intake from food and fluids was significantly higher in those rats that consumed the 20% fructose solution, despite consuming less food. These findings corroborate with a previous study that observed no significant difference in terminal body mass despite having a significantly increased caloric intake from high fructose diet (20% fructose solution) feeding, for a period of 90 days (Ramos et al., 2017). The reduction in food intake in those rats that consumed the fructose solution was compensated for by the increased consumption of the fructose solution, as was demonstrated by the increase in total caloric intake (Table 3.2). However, the anticipated increase in terminal body mass from the high fructose diet due to the increased total caloric intake was not observed in the current study. Body mass and other anthropometric measurements such as BMI and the Lee index (not measured in the current study) can be used as a proxy for the assessment of growth performance and to an extent, adiposity (Li et al., 1998, Metwally et al., 2019). However, these measurements do not give an indication of body composition (lean mass vs fat mass) and the location of the fat depots, therefore it is better in terminal studies, to directly measure the visceral fat depots (Metwally et al., 2019).

4.3. Visceral Fat Mass

Previous studies have shown that despite similar body weights to their counterparts on standard rat chow, rats which had *ad libitum* access to 20% fructose solution to drink, as was the case in the current study, had significantly increased visceral fat deposits (Ramos et al., 2017, Lembede et al., 2018, Gumede et al., 2020a, Gumede et al., 2020b). Ramos et al. (2017), speculated that the high adipogenic nature of

fructose was responsible for the significantly increased visceral fat depots without a commensurate increase in body mass in rats consuming a 20% fructose solution. In an earlier study, diet induced obesity was shown to be reduced by quercetin administration. Quercetin reduced the obesity associated insulin resistance and oxidative stress, by ameliorating the diet-induced adipose tissue macrophage infiltration and inflammation. Quercetin also upregulated adenosine monophosphate activated protein kinase α 1 phosphorylation and the expression of silent information regulator 1in adipose tissues, subsequently reducing adipocyte tissue size and body fat mass (Dong et al., 2014).

However, the fructose induced visceral adiposity in the current study was not prevented by the concurrent administration of quercetin. Our findings are the similar to those of a previously reported study in which quercetin was administered, at 100mg/kg body weight, together with a 20% fructose solution for 10 weeks to growing Sprague Dawley rats. The authors observed increased visceral fat deposits in the rats receiving the fructose solution compared to the controls, and the obesity was not prevented by the quercetin administration (Molopo et al., 2019). Thus considering the findings of Dong et al. (2014) that used a high fat diet with quercetin in comparison to that of Molopo et al (2019), and the current findings. The quercetin used in both high fat diets and fructose diet induced metabolic dysfunction seems to be more effective when used as a treatment than as a prophylaxis for obesity (Dong et al., 2014, Molopo et al., 2019).

Visceral obesity is directly connected with undesirable metabolic outcomes such as insulin insensitivity, disturbances in glycaemic control and lipid metabolism, with consequent hypertriglyceridemia, hypercholesterolemia, and low serum adiponectin levels (Agostinis-Sobrinho et al., 2020).

4.4. Serum Insulin, Adiponectin, Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) Index and Circulating Metabolites

Previously, studies have linked a high fructose diet with metabolic dysfunction including hyperglycaemia, hyperinsulinemia, insulin resistance, elevated HOMA-IR index, dyslipidaemia, and decreased serum adiponectin level (Anvari et al., 2014, Lustig et al., 2016, Elshazly et al., 2020, Hieronimus and Stanhope, 2020). In contrast to previous studies, in this study, the high fructose did not affect serum FBG, fasting insulin, HOMA-R index, adiponectin, TC and LDL-C. Whereas HDL-

C concentration were significantly increased in those rats that received fructose together with quercetin, compared to the rats in the negative control group. This finding is nevertheless consistent with earlier studies, that reported elevated levels of plasma HDL-C following concomitant administration of a high fructose solution with quercetin to rats (Jian-Mei et al., 2008, Sikder et al., 2014, Abdelkarem and Fadda, 2017, Cui et al., 2017, Lu et al., 2017).

HDL-C is considered a 'good' lipoprotein and is beneficial to healthy living because of its crucial role in cholesterol metabolism and homeostasis (Ren et al., 2017). The molecular mechanisms through which quercetin elevates plasma HDL-C were not assessed in the current study. Nonetheless, in an earlier study, it was suggested that quercetin increased plasma HDL-C concentration by upregulating the expression of the hepatic scavenger receptor class B type I and scavenger receptor class B-I mediated selective lipid uptake, through activation of the proliferator-activated receptor gamma and liver X receptor alpha signalling pathway (Ren et al., 2017). In another study, it was reported that consumption of a diet rich in quercetin increases plasma HDL-C levels by increasing plasma apolipoprotein A1 concentrations (Khadem-Ansari et al., 2010). However, based on the current study, I can speculate that quercetin increased the serum HDL-C level better when consumed together with fructose.

Failure of the 20% fructose solution to induce some of the expected metabolic dysfunction (hyperglycaemia, dyslipidaemia, and insulin resistance) in the current study is in agreement with an earlier study. That also observed no differences in serum fasting blood glucose concentrations, insulin concentrations, the HOMA-IR index and total cholesterol at the end of 10 weeks of feeding growing female Sprague Dawley rats a diet high in fructose (20% fructose solution) (Muhammad et al., 2019). The inconsistencies of the current study results with respect to other previous studies that reported hyperglycaemia, insulin resistance, decreased serum adiponectin levels and dyslipidaemia following high fructose diet (20% fructose solution) feeding, could be due to the sex of the rats used in this study. Previous studies have shown that the female sex hormone (oestrogen) may have some protective effects against metabolic dysfunction induced by high fructose feeding (Galipeau et al., 2002, Couchepin et al., 2008). Song and his co-workers observed that gonadectomised male rats fed a 60% fructose solution for 63 days, exhibited less fructose mediated metabolic dysfunction in comparison with their male counterparts with intact gonads (Song et al., 2004). Additionally, epidemiological

studies have reported that there is a greater tendency for high fructose feeding to cause metabolic dysfunction in postmenopausal women, compared to men of the same age (Lonardo et al., 2019, Yuan et al., 2019).

The lack of development of some of the metabolic dysfunction associated with fructose consumption in this study might be due to the age of the rats. Because in the current study, most part of our intervention corresponds to periods of rapid growth and development, which have higher energy demands compared to that in adulthood. Therefore, the high fructose diet (20% fructose solution) used in the current study could have failed to induce significant metabolic dysfunction because the energy from the 20% fructose solution was possibly being used for this phase of development (Iossa et al., 1999). De Moura and his colleagues, observed that adult rats (90 days old) were less protected from the negative effects of fructose in comparison to younger rats (28 days old), following high fructose feeding, suggesting unique protective mechanisms in the younger rats against the negative effects of high fructose consumption (De Moura et al., 2009). The liver is central to metabolism and high fructose diet induced NAFLD can occur independently of general metabolic dysfunction (Muhammad et al., 2017, Nyakudya, 2018). The next section will focus on the hepatic lipid yield, liver mass and histopathology in detail.

4.5. Hepatic Lipid Yield, Liver Mass, Hepatosomatic Indices and Liver Histology

4.5.1. Hepatic lipid yield, liver mass, and hepatosomatic indices

High fructose diets are linked to increased, uncontrolled hepatic fructolysis, lipid formation and increased lipid accumulation consequently causing NAFLD (Tappy and Le, 2010, Hannou et al., 2018, Loza-Medrano et al., 2020). In the current study, hepatic lipid yield, liver mass and hepatosomatic indices were increased by the fructose (20% fructose solution) consumption. This finding indicated that consumption of the 20% fructose solution for 12 weeks resulted in increased hepatic lipid deposition which can potentially advance from simple liver steatosis to NASH, hepatic fibrosis, or hepatocellular carcinoma. Similar results were seen in rats following 20% fructose solution feeding for 57 and 70 days (Mamikutty et al., 2015, Muhammad et al., 2019).

In this study, the quercetin supplementation failed to ameliorate the increase in hepatic lipid yield, liver mass, and hepatosomatic index of those rats that consumed

high fructose diet together with the quercetin. These results are in agreement with a previous study that reported quercetin's failure to reduce liver mass and hepatic lipid content at the end of 10 weeks of treatment with 20% fructose solution and quercetin (Molopo et al., 2019). Although quercetin supplementation in the present study did not significantly reduce the total hepatic lipid content, there was a trend towards the reduction of hepatic lipid yield from 13.44 ± 3.44% (in the fructose alone group) to 9.58 ± 2.73% (in the fructose and quercetin group), which corresponds to a 29% hepatic lipid yield reduction. The 29% reduction in liver lipid content observed in our study can be considered biologically or clinically relevant, because the hepatic lipid content following quercetin treatment is below the 10% mean value cut off mark for diagnosis of NAFLD (Vanni et al., 2010, Marchisello et al., 2019).
Furthermore, the increased liver lipid deposition we recorded in the present experiment could be responsible for the increase in liver mass and hepatosomatic index as suggested in a previous study, presumably not due to quercetin treatment or liver damage (Rippe and Angelopoulos, 2013). In the current study, only the liver lipid content of the rats that consumed fructose was higher than the 10% cut off mark considered for the diagnosis of NAFLD. However, determination of total liver lipid content alone is inadequate for the thorough diagnosis and staging of NAFLD, since it cannot evaluate other histopathological features of NAFLD such as lobular inflammation, ballooning, and fibrosis (Nasr et al., 2020). Consequently, the histological assessment of liver samples is regarded as the benchmark investigation for the diagnosis of NAFLD (Marchisello et al., 2019).

4.5.2. Liver histology (hepatic steatosis, inflammation, and fibrosis)

Similar to earlier research by Nyakudya and colleagues histomorphological assessment of the liver samples in the current study revealed that 20% fructose solution feeding resulted in the development of hepatic steatosis, which might ultimately progress to NASH and hepatic fibrosis (Nyakudya et al., 2018). All the histopathological changes observed following consumption of the 20% fructose solution were attenuated by the quercetin treatment. These findings are in line with a previous study done on growing male Sprague Dawley rats, that reported improved hepatic steatosis and inflammation following concurrent administration of 20% fructose solution and quercetin for 10 weeks (Molopo et al., 2019). Another earlier study reported that quercetin improved hepatic steatosis, inflammation, and fibrosis

by suppression of hepatic stellate cell activity and limiting autophagy through TGF-β1/Smads and PI3K/Akt pathways (Wu et al., 2017).

In my present study, the molecular mechanisms through which quercetin may have exerted its hepatoprotective effects were not assessed, however, earlier studies reported that quercetin treatment decreases hepatic steatosis by enhancing the genes associated with mitochondrial biogenesis and oxidative metabolism in lipid-laden hepatocytes (Kim et al., 2015). This, along with enhanced levels of the transcription factor, nuclear erythroid 2-related factor 2, and heme oxygenase-1 protein, protects against inflammatory and oxidative stress, as well as metabolic dysregulation (Kim et al., 2015, Nettore et al., 2019). Moreover, quercetin was reported to prevent oxidative stress, inflammation and limit hepatic fibrosis by decreasing pro inflammatory cytokine and liver cell apoptosis. These effects were perceived to be accomplished through the inhibition of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, p38 mitogen-activated protein kinase, and B-cell lymphoma protein 2 associated X and Bcl-2 signalling pathways. Thereby, improving hepatic oxidative stress and inflammatory status, reducing hepatocyte apoptosis and thus improving the high fructose induced liver damage (Wang et al., 2017). The present study confirms the importance of histological assessment for NAFLD rather than use of total lipid content determination.

Liver damage, due to excessive lipid deposition (NAFLD) resulting from a high fructose diet, is often reported to coexist with a similar condition in the pancreatic tissue (non-alcoholic fatty pancreas disease - NAFPD). NAFPD often leads to increases in pancreatic mass and the pancreatosomatic index, as well as histopathological changes within the pancreas upon biopsy (Yu and Wang, 2017, Rosenblatt et al., 2019). The results pertaining to the pancreatic mass, pancreatosomatic index and pancreatic histology will be discussed in detail in the next section.

4.6. Pancreatic Mass, Pancreatosomatic Indices and Pancreatic Histology

4.6.1. Pancreatic mass, pancreatosomatic indices

High fructose diet consumption has been associated with metabolic dysfunction including hypertriglyceridaemia, insulin resistance, type 2 diabetes, increased proinflammatory cytokine production and increased inflammatory cell infiltration. These metabolic events can lead to increased lipid deposition in the pancreas

(NAFPD), as well as inflammation and oxidative stress (which might result in steatopancreatitis and pancreatic fibrosis), which if left untreated might progress to pancreatic cancer, consequently increasing pancreatic mass and the pancreatosomatic index (Li et al., 2013, Topsakal et al., 2016). In the current study, neither the pancreatic mass, nor the pancreatosomatic indices were significantly affected by the high fructose consumption or treatment with quercetin, suggesting insignificant pancreatic parenchymal damage, if any.

4.6.2. Pancreatic histology (pancreatic steatosis, inflammation and fibrosis)

Histological assessment of pancreatic tissue, which is the gold standard diagnostic tool for diagnosis of NAFPD, was done to further assess the pancreas for NAFPD. Histological assessment of the pancreas demonstrated insignificant pancreatic lipid deposition within all treatment groups, far below the minimal threshold (7% lipid deposition per microscopic field at 40x) considered for the diagnosis of NAFPD (Catanzaro et al., 2016). Additionally, in this present experiment no pancreatic inflammation or fibrosis was observed in any of the treatment groups. Thus, in the current study, the 20% fructose solution did not induce the anticipated histopathological changes associated with NAFPD (pancreatic steatosis, inflammation, and fibrosis) within the pancreas of the rats. Previous studies have linked the extent of injury/damage to organs such as the liver, pancreas and kidneys to the serum concentrations of specific markers of organ function (Muhammad et al., 2019, Tajima et al., 2019a). Hence, the serum biomarkers of general health and liver, pancreas and renal function were measured in the current study and will be discussed in detail in the next section.

4.7. Red Blood Cell Indices, and markers of Hepatic, Renal, and Pancreatic Function

When conducting an experimental animal study on any potential drug agents, including synthetic pharmacological agents' or phytomedicine agents, it's important to evaluate the agent's potential toxic effects on the red blood cell indices, as well as on the liver, kidneys and pancreas. These organs are essential in drug metabolism and detoxification of any hazardous substances administered to experimental animals, as well as in the general health status of the animals (Alebachew et al., 2014, Yadav et al., 2020). The general health status of the rats in the current study

was further assessed by evaluating the red blood cell indices (PCV, Hb and MCHC) at the end of the12 week feeding period. It is a known fact that both high fructose feeding and NAFLD are associated with iron deficiency and exaggerated cellular oxidative stress, which might cause red blood cell destruction and death, eventually leading to anaemia, decreased PCV, Hb, and MCHC (Mihafu et al., 2020, Pasdar et al., 2020). However, the PCV, Hb and MCHC between the groups in the current study were not different, most likely due to the mild to moderate presentation of the metabolic dysfunction in the rats.

Hepatic function was assessed through the measurement of blood alanine aminotransferase (ALT), total bilirubin and albumin concentrations. ALT is a hepatic cytosolic enzyme that is released into the systematic circulation when the hepatic tissue is damaged, such as in NAFLD, resulting in an elevated plasma ALT concentration (Botezelli et al., 2012). Additionally, hepatic tissue damage has been associated with elevated plasma total bilirubin and decreased plasma albumin concentrations, due to decreased hepatic clearance of bilirubin and impaired albumin production, respectively (Abdul-Mounther et al., 2016, Singha et al., 2017). Hence, the use of plasma concentrations of alanine aminotransferase, total bilirubin and albumin to evaluate liver function (surrogate markers of liver function) of studied animals in current study.

Kidney function was ascertained by measuring serum creatinine and blood urea and nitrogen (BUN) concentrations. The gold standard for assessing kidney function involves measurement of the glomerular filtration rate (GFR). However, GFR is difficult to measure clinically, hence our use of serum creatinine and blood urea nitrogen (BUN) concentrations, and the BUN/Creatinine ratio, as proxy indicators of renal function (Delanaye et al., 2020). Impaired renal function has been strongly correlated with elevated serum creatinine, BUN, and BUN/Creatinine ratio (Kamal, 2014). Previous studies have shown high fructose diet consumption to be linked with raised serum concentrations of biomarkers of general health and hepatic and renal function, due to the resulting fructose-induced nephropathy and hepatocellular injury/damage (Ozawa et al., 2016, Cheng et al., 2020, Kim and Min, 2020).

Even though the fructose feeding led to the development of NAFLD in the current study, which was reduced by the quercetin supplementation,neither the 20% fructose solution, nor the quercetin significantly affected any of the markers of general health, hepatic or renal function. These findings are in agreement with Molopo’s study with 20% fructose solution and quercetin at 100 mg/kg body in growing male Sprague

Dawley rats for 10 weeks, that reported no significant differences in markers of general health, renal or hepatic function (Molopo et al., 2019). The lack of differences in markers of general health and renal and hepatic function could possibly be due to the NAFLD being in the early stages. Thus, there was probably no significant hepatocyte destruction as yet and consequently no notable elevation in the plasma biomarkers assessed (Palekar et al., 2006, Gumede et al., 2020b). Furthermore, several earlier studies suggest that for a significant increase in the concentration of biomarkers of organ function to be detected in the system, the organ damage/injury has to be greater than fifty percent (50%) (Ozer et al., 2008, Antoine et al., 2009, Muhammad et al., 2019, Tajima et al., 2019b). The orally administered quercetin did not compromise the liver or renal function, nor general health profile of the rodents in the current study. Therefore, quercetin could possibly be used as a potential prophylactic agent against fructose induced steatosis, inflammation and fibrosis in organs such as the liver and pancreas.

In the current study pancreatic function was assessed by determining the pancreatic enzymes (lipase and amylase) level in the plasma. Lipases are enzymes that catalyse the breakdown of long-chain fatty acids into β-monoglycerides and two free fatty acids. Lipases are identified in a variety of tissues such as the pancreas, intestine, leukocytes, lung, adipose tissue and liver. However, the concentration of lipase is extremely high in the pancreas (≈ 9000 times) compared to other tissues (Tietz and Shuey, 1993, Cotten, 2020). Amylases are enzymes produced by various tissues such as the pancreas, salivary glands, adipose tissues, fallopian tubes, striated muscles and liver, they accelerate the hydrolysis of starch and glycogen into glucose (Janowitz and Dreiling, 1959, Mcgeachin and Lewis, 1959).

Like previous studies, in the current study the plasma levels of pancreatic enzymes (lipase and amylase) were used as a surrogate marker of pancreatic inflammation (pancreatitis) and fibrosis. Although, elevated levels of plasma lipase and amylase are crucial signs for the diagnosis of pancreatic inflammation and fibrosis, the plasma lipase concentration has superior clinical sensitivity because lipase activities remain increased for longer (8 to14 days) periods of time than those of amylase (Janowitz and Dreiling, 1959, Tietz and Shuey, 1993). In the current study, the plasma amylase concentration was significantly more in the rats that consumed the high fructose solution compared to the rats in the negative control or quercetin only groups. The plasma lipase concentration was not different between treatment groups, suggesting that the elevated plasma amylase in this study was presumably

not a result of pancreatic damage, since histological examination of the pancreas, which is the benchmark tool for the diagnosis of NAFPD, revealed that the 20% fructose treatment did not induce the anticipated pancreatic steatosis, inflammation, or fibrosis. The elevated serum amylase could be due to fructose mediated amylase production, secondary to high fructose diet consumption as reported in earlier research (Howard and Yudkin, 1963, Christophe et al., 1971, Azzout-Marniche et al., 2018). However, the elevated level of plasma amylase could have originated from other sources (such as salivary glands, ovaries, fallopian tubes, lungs, striated muscles, or adipose tissue), other than the pancreas alone (Cotten, 2020). The total amylase activity in plasma is formed by pancreatic amylase (P-type or AMY2) and salivary amylase (S-type or AMY1) isoenzymes. Therefore, evaluation of specific isoenzymes present in the circulation will provide better information on enzyme changes during disease development than total activities alone, hence increasing diagnostic precision (Ismail and Bhayana, 2017).

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Given the burden that diet induced metabolic disorders place on health care systems worldwide, and the lack of holistic prophylactic medical interventions. We hypothesized that dietary enrichment with the phytochemical quercetin might offer protection against the development of metabolic dysfunction (including NAFLD and NAFPD), after 84 days of *ad libitum* ingestion of 20% fructose solution by growing female Sprague Dawley rats, from the early post weaning period. The findings of this study showed that the consumption of a 20% fructose solution significantly increased visceral adiposity, liver mass, hepatosomatic index and hepatic lipid yield, while reducing serum HDL-C. However, when quercetin was given concurrently with 20% fructose solution, quercetin was able to increase the HDL-C level and reduce hepatic lipid yield, but failed to improve the visceral fat mass, liver mass and hepatosomatic index. Considering the unreliability of gross measurements of the liver in the assessment of NAFLD, histological assessment ratified that the orally consumed 20% fructose solution for 12 weeks to growing female Sprague Dawley rats resulted in the development of NAFLD, which was improved significantly with quercetin treatment.

As we have observed in previous studies using young rats, the 20% fructose solution treatment did not cause the anticipated hyperglycaemia, hypertriglyceridaemia, hypercholesterolaemia, insulin resistance or obesity. Additionally, there was no development of metabolic dysfunction within the pancreas i.e. non-alcoholic fatty pancreas disease (NAFPD). Hence, for overt metabolic dysfunction development relying on the results of this research we can speculate that possibly a longer period of treatment (longer than 12 weeks) or a higher concentration of fructose solution might be required to induce NAFPD in the growing female Sprague Dawley rats. Thus, it seems that in female rats, signs of NAFLD can appear before those of NAFPD and this need to be factored into future studies. As noted in the rationale for the study where previous studies have focussed on males, we found that the dietary quercetin supplementation was well tolerated by the growing female rats in the current study, without any observed undesirable negative effects on the measured parameters. Factoring in the positive health outcomes observed in the fructose fed female rats; this shows that quercetin can also be used in female rats for its metabolic health benefits.

5.2. Limitations and Recommendations

In the current study, consumption of the 20% fructose solution *ad libitum* resulted in development of hepatic steatosis, inflammation and fibrosis, however, there were no other signs of metabolic dysfunction, as evidenced by the unchanged circulating biomarkers of health and the lack of fructose induced changes in the pancreas (NAFPD) of growing female Sprague Dawley rats, possibly due to it not providing enough excess calories which might consequently cause metabolic dysfunction. Therefore, future studies, using younger rats should consider using more prolonged period of fructose consumption or higher concentration fructose solution in order to induce more significant metabolic dysfunctions including NAFPD.

In future, the fructose should probably be delivered via the feed rather than in the drinking water, since earlier research has proved it to be more powerful in stimulating the required metabolic dysfunction such as NAFLD, NAFPD.

One of the limitations of this study is that total plasma amylase activity which presents both salivary and pancreatic amylase was quantified and used to evaluate the pancreatic function. Therefore, advisedly the future studies should specifically quantify the pancreatic amylase for better or precise evaluation of pancreatic function.

Another limitation of the current study was that molecular studies which might define the mechanisms invoked by the quercetin to prevent the high fructose induced NAFLD by measuring the gene and protein expression were not done. Therefore, subsequent studies should employ molecular techniques to determine some of the mechanisms responsible for the protective effect of quercetin against NAFLD.

CHAPTER 6: REFERENCES

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APPENDICES

Appendix A: AESC Clearance Certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/02/07/B

APPLICANT: Dr J Donaldson

SCHOOL: School of Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups

Number and Species

192 each male and female postnatal day 7, 36 female postnatal day 21 and 40 female nursing adult Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/02/28. This approval remains valid until 2019/03/15.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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

None

Signed: _____ (Chairperson, AESC) Date: 22/03/2017

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

Signed: _____ (Registered Veterinarian) Date: 19/03/17

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

Appendix B: Ethics Clearance Modification and Extensions Certificate

AESC 2019

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Janine Donaldson

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO: 2017/02/07/B**

Other M&E's : 3

d. Project Title: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups.

e.	Number and species of animals **originally approved**:		192 male and 192 female Sprague Dawley rat pups-postnatal day 7; 36 female Sprague Dawley rat pups-postnatal day 21; 40 female nursing adult Sprague Dawley rats
f.	Number of additional animals **previously allocated on M&Es**:		N/A
g.	Total number of animals **allocated** to the experiment to date:		76 female Sprague Dawley rat pups-postnatal day 21 and 78 male Sprague Dawley rat pups-postnatal day 21
h.	Number of animals **used** to date:		76 female Sprague Dawley rat pups-postnatal day 21 and 78 male Sprague Dawley rat pups-postnatal day 21

i. Specific modification / extension requested:

- We would like to **request for an extension on the ethics approval** granted in March 2017, which is valid until 15th March 2019.
- We would like to **include the following people as co-workers** on the project:
  - Abubakar Namadina (student number: 1965524)

j. Motivation for modification / extension:

- We have conducted various experiments under the ethics approval granted in 2017 (see progress report document attached outlining experiments carried out to date, along with original ethics approval certificate and subsequent approved M & E's) and would like to continue with the research on quercetin and fructose-induced metabolic abnormalities.

- The extension in time will enable us to continue with the metabolic programming studies (outlined as Experiment 1 and 2 in the original ethics application), where the potential of neonatal quercetin supplementation in theprotection against development of metabolic dysfunction as a result of exposure to high-fructose diets,both during the neonatal period, adolescence and again later in adulthood, will beinvestigated.
- Dr Namadina will be using the data collected from the above mentioned experiment as part of his MSc degree

Signature: Date: 11/03/2019

Dr Janine Donaldson

RECOMMENDATIONS:

- Extension of time until 15th of March 2021.
- Addition of Abubakar Namadina. – requirement: to attend CAS orientation course.

Signature: _____ Date: 11/03/2019

Chairman, AESC pp.

Appendix C: Manufacturer Instructions for Rat INS (Insulin) ELISA Kit

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 Conjugate working 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.

Appendix D: Manufacturer Instructions for Rat ADP/Acrp30 (Adiponectin) ELISA Kit

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 Conjugate working 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.

Appendix E: Manufacturer Instructions for Total Cholesterol (TC) Assay Kit (single reagent, COD-PAP method)

Operation (procedure) Steps /Colorimetric assay with Spectrophotometer.

1. Bring all the reagents and samples to room temperature and allow the samples to thaw before use.
2. Centrifuge the samples again after thawing before the assay.
3. All the reagents should be mixed thoroughly by gently swirling before pipetting and avoid foaming.
4. All samples and standards to be assayed in duplicates.
5. Add 10 µL of distilled water, standard cholesterol and samples to Blank, Standard and Samples test tubes respectively.
6. Add 1000 µL of working solution to all the Blank, Standard and Samples test tubes.
7. Mix the mixture from (6) above fully and incubate at 37 °C for 10 minutes.
8. Set the spectrophotometer to zero with double distilled water.
9. Measure the optical density (OD) value of each tube at 510 nm wavelength with 0.5 cm diameter cuvette using spectrophotometer.
10. After the experiment, put all the unused reagents in refrigerator according the specified storage temperature until their expiry date.

Calculation formula of serum

TC Content (mmol/L) = (OD Sample – OD Blank) / (OD Standard – OD blank) * Concentration of standard (5.17 mmol/L).

Appendix F: Manufacturer Instructions for Low-density Lipoprotein Cholesterol (LDL-C) Assay Kit (Double reagents)

Assay procedure/Colorimetric assay with Spectrophotometer.

1. Bring all the reagents and samples to room temperature and allow the samples to thaw before use.
2. Centrifuge the samples again after thawing before the assay.
3. All the reagents should be mixed thoroughly by gently swirling before pipetting and avoid foaming.
4. All samples and standards to be assayed in duplicates.
5. Add 10 µL of distilled water, standard cholesterol and samples to Blank, Standard and Samples test tubes respectively.
6. Add 750 µL of Reagent 1 to all the Blank, Standard and Samples test tubes.
7. Mix the mixture from (2) above fully and incubate at 37 °C for 5 minutes.
8. Set the spectrophotometer to zero with double distilled water.
9. Measure the optical density (OD) value (A1) of each tube at 546 nm wavelength with 0.5 cm optical path cuvette in spectrophotometer.
10. Add 250 µL of reagent 2 to all the Blank, Standard and Samples test tubes.
11. Mix the mixture from (6) above fully and incubate at 37 °C for 5 minutes.
12. Set the spectrophotometer to zero with double distilled water.
13. Measure the optical density (OD) value (A2) of each tube at 546 nm wavelength with 0.5 cm optical path cuvette in spectrophotometer.
14. After the experiment, put all the unused reagents in refrigerator according the specified storage temperature until their expiry date.

Calculation formula of serum

LDL-C Content (mmol/L) = (A2sample – A1sample) – (A2blank – A1blank) / (A2standard – A1standard) – (A2blank – A1bank) * Concentration of standard (2.4 mmol/L) for LDL-C.

Appendix G: Manufacturer Instructions for High-density Lipoprotein Cholesterol (HDL-C) Assay Kit (Double reagents)

Assay procedure/Colorimetric assay with Spectrophotometer.

1. Bring all the reagents and samples to room temperature and allow the samples to thaw before use.
2. Centrifuge the samples again after thawing before the assay.
3. All the reagents should be mixed thoroughly by gently swirling before pipetting and avoid foaming.
4. All samples and standards to be assayed in duplicates.
5. Add 10 µL of distilled water, standard cholesterol and samples to Blank, Standard and Samples test tubes respectively.
6. Add 750 µL of Reagent 1 to all the Blank, Standard and Samples test tubes.
7. Mix the mixture from (2) above fully and incubate at 37 °C for 5 minutes.
8. Set the spectrophotometer to zero with double distilled water.
9. Measure the optical density (OD) value (A1) of each tube at 546 nm wavelength with 0.5 cm optical path cuvette in spectrophotometer.
10. Add 250 µL of reagent 2 to all the Blank, Standard and Samples test tubes.
11. Mix the mixture from (6) above fully and incubate at 37 °C for 5 minutes.
12. Set the spectrophotometer to zero with double distilled water.
13. Measure the optical density (OD) value (A2) of each tube at 546 nm wavelength with 0.5 cm optical path cuvette in spectrophotometer.
14. After the experiment, put all the unused reagents in refrigerator according the specified storage temperature until their expiry date.

Calculation formula of serum

HDL-C Content (mmol/L) = (A2sample – A1sample) – (A2blank – A1blank) / (A2standard – A1standard) – (A2blank – A1bank) * Concentration of standard (1 mmol/L) for HDL-C.

Appendix H: Soxhlet Procedure for Lipid Extraction (Liver samples)

Soxhlet Procedure for Lipid Extraction

Hepatic lipids extraction was carried out using Manual Soxhlet Apparatus (procedure), as outline below.

Procedure steps;

1. Set all the Soxhlet apparatus parts together with samples and the extraction solvent to be used (petroleum ether in this case).
2. Soak the extraction thimble in the petroleum ether and placed the oil free cotton wool in the thimble.
3. The material to be extracted (0.5 g of Homogenized Hepatic sample) is placed inside the thimble over the oil free cotton wool.
4. Then load the thimble with sample into the main chamber of the Soxhlet extractor.
5. Weigh the empty distillation flask and note the weight.
6. Add 200 ml of petroleum ether in the distillation flask and place it on the heating element.
7. Place the Soxhlet extractor atop the flask and ensure they are fitted.
8. Place the reflux condenser atop the Soxhlet extractor and ensure they are fitted.
9. Set the thermostat of the heating element at 50± 10°C (boiling point of petroleum ether).
10. Turn on the cooling water supply to the condensers and Switch on the power supply to the heating element.
11. Allow the procedure to run for 2 hours and switch off the power and water supply.
12. Remove the thimble from the extractor, and pour the remaining ether into the distillation flask.
13. Evaporate the petroleum ether on a rotator evaporator at 50 ± 10°C until all the ether evaporated leaving only the oil in the flask.
14. Allow the flask to cool and Weigh the flask containing the oil, and note the weight.

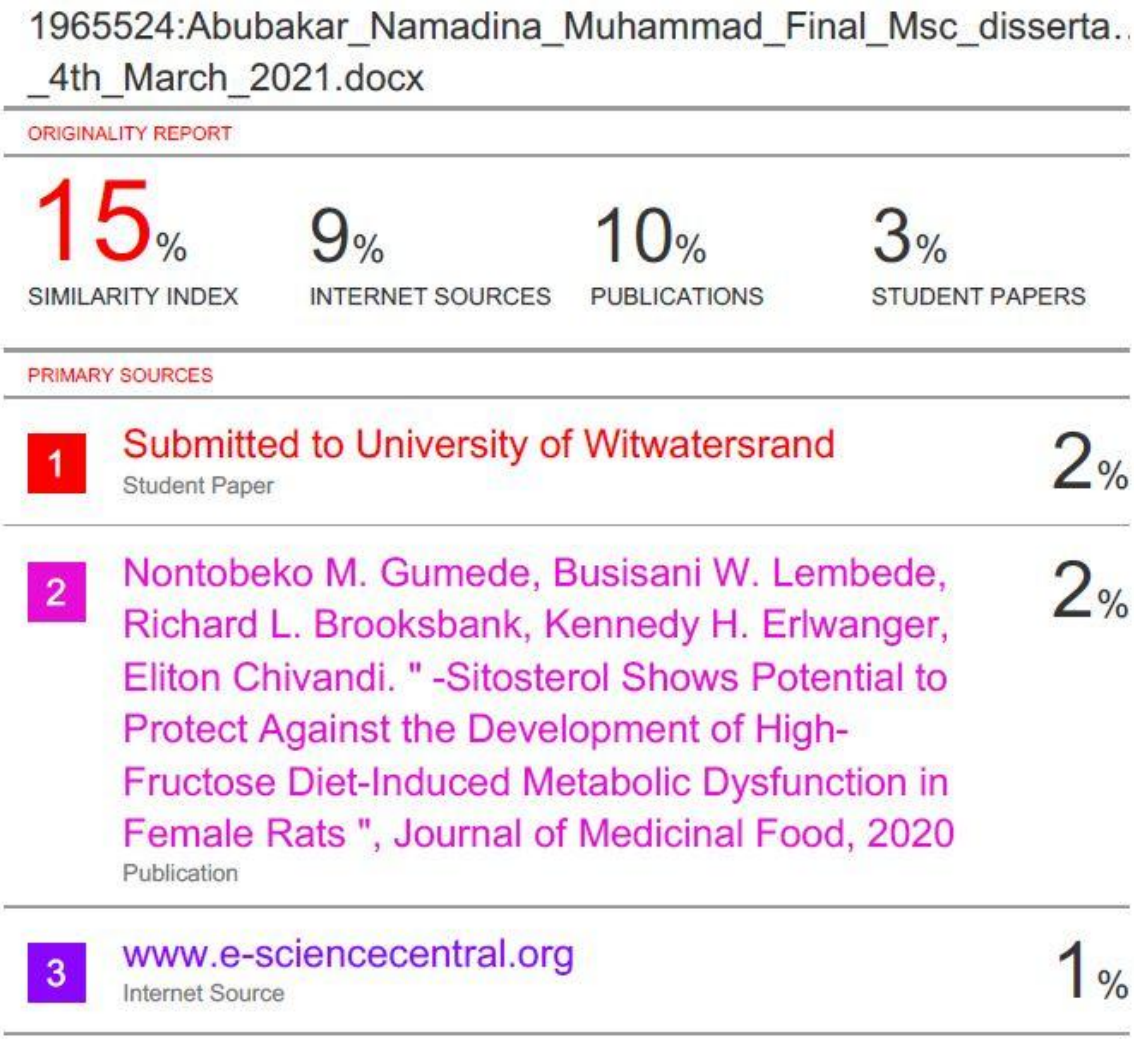
Calculation of the Extracted Fat (%):

$$\% \text{ fat} = \frac{\text{Weight of flask with oil} - \text{weight of empty flask}}{\text{Weight of liver sample}} \times 100$$

Storage:

Add a little petroleum ether to the oil and store at 4 ± 2°C.

Appendix I: Turnitin Report



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