INVESTIGATING THE METABOLIC AND ANTIOXIDATIVE PHARMACOLOGICAL POTENTIAL OF ELLAGITANNINS OBTAINED FROM POMEGRANATE FRUIT PEEL IN FRUCTOSE-FED EXPERIMENTAL RATS

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Central University of Technology, Free State

DECLARATION OF INDEPENDENT WORK

I, Nomonde Patience Mapasa, student number _____, hereby declare that this research project, submitted to the Central University of Technology, Free State for the degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY, is my own independent work. It complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted, either in full or in part, to any other institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification. As such, where external sources were utilised, due acknowledgment was given by means of a comprehensive list of references in accordance with departmental requirements. I therefore give copyright of this dissertation to the Central University of Technology, Free State.

Candidate: Nomonde P. Mapasa — 24 September 2025 Date

Supervisor: Dr Chika I. Chukwuma — 24 - 09 - 2025 Date

Co-supervisor: Dr Claudia M. Ntsapi — 24/09/2025 Date

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A special word of gratitude to Linda, who went beyond the call of duty, thank you.

DEDICATION

I dedicate this work to God.

"**Let the morning bring me word of your unfailing love, for I have put my trust in you. Show me the way I should go, for to you I entrust my life.**" – Psalms 143:8

To my family, with love and gratitude.

ABSTRACT

Background

The increasing prevalence of obesity, and some unhealthy lifestyle factors such as physical inactivity and unhealthy dieting, has exacerbated the prevalence of metabolic and cardiovascular diseases. Specifically, chronic consumption of added sugars such as sucrose and fructose has been implicated in metabolic and cardiovascular impairments, including insulin resistance and dyslipidaemia. Pomegranate fruit is a fruit that is beneficial to oxidative and vascular health, largely attributed to its constituent ellagitannins. Increasing evidence indicates that the peel is particularly rich in ellagitannins and possesses antioxidant, glycaemic control, and anti-dyslipidaemic potentials. However, the ameliorative potential of pomegranate peel or its ellagitannins on metabolic disorders associated with chronic consumption of added sugars remains poorly investigated or understood. Therefore, this study was undertaken to investigate the effect of an ellagitannins-rich fraction obtained from pomegranate peel on selected metabolic alterations in rats induced by high fructose consumption.

Methodology

Class 1 grade of pomegranate fruit (Var. “Wonderful) was bought from a local fruit packaging company (Sonlia Fruit Packhouse, Wellington, Western Cape Province, South Africa). The peel was removed and extracted with distilled water to obtain the crude water extract. An ellagitannins-rich fraction was obtained from the crude extract using an Amberlite® XAD16N resin. The phytochemicals in the fractions were determined and quantified using liquid chromatography-mass spectrometry (LC-MS). For the animal experiment, eighteen 14 weeks old Sprague-Dawley rats were procured and allowed to acclimatize for 1 week. The animals were then divided into three groups, namely “Normal Control”, “Fructose”, and “Fructose + Ellagitannins” groups. The “Normal Control” received normal drinking water. The “Fructose” group received 20% fructose solution instead of drinking water. The last group, “Fructose + Ellagitannins” received 20% fructose solution containing 200 mg/kg body weight of the fraction in place of drinking water. The treatments lasted for 6 weeks, during which body weight and food intake were measured weekly. In the last week of the experiment, oral glucose tolerance test (OGTT) was carried out, while fasting (FBG) and non-fasting (NFBG) blood glucose were measured. At the end of the 6-week experimental period, the blood lipids (triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol) levels were measured.

The animals were then euthanized, and blood and liver tissue were collected. Serum and liver homogenates were prepared from the blood and liver tissue, respectively. Liver glycogen content and lipid peroxidation level was determined, while serum insulin, liver total and phospho Akt, and liver interleukin 1β (IL-1β) levels were measured using commercial ELISA kits. Catalase and superoxide dismutase (SOD) activities were measured using commercial colorimetric assay kits. Liver histology was examined microscopically, and the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated.

Results

Fructose consumption suppressed food intake but increased weight gain. It also elevated insulin levels, impaired glucose tolerance (AUC = 246 vs 208 mg.h/dL), reduced insulin sensitivity (HOMA-IR = 5.8 vs 2.0), and altered blood lipid profiles (triglycerides = 4.2 vs 1.7 mmol/L; LDL-cholesterol = 3.0 vs 1.4 mmol/L; HDL-cholesterol 0.4 vs 0.8 mmol/L). In addition, it caused vacuolar hepatopathy, induced hepatic inflammation (IL-1β elevation), and increased both hepatic and systemic oxidative stress. Treatment with ellagitannins treatment improved glucose tolerance (AUC = 230 mg.h/dL), insulin resistance (HOMA-IR = 4.0; with improved hepatic Akt phosphorylation), blood lipids (triglycerides, LDL-cholesterol and HDL-cholesterol = 3.7, 1.9, and 0.6 mmol/L, respectively), hepatic histology, inflammation, and antioxidant status (reduced lipid peroxidation and increased catalase and SOD enzyme activity). LC-MS showed the presence of bioactive ellagitannins (punicalagin, corilagin, and pendunculagin) and catechins, with punicalagin being the most predominant.

Conclusion

Ellagitannins from pomegranate peel exerted ameliorative effects on fructose-induced metabolic alterations, such as impaired glycaemic control, dyslipidaemia, inflammation and oxidative stress in rats. Ellagitannins from pomegranate peels may therefore be useful dietary polyphenols capable of preventing or managing sugar-induced metabolic, cardiovascular, and oxidative disorders.

Keywords: *Pomegranate peel, ellagitannins, metabolic syndrome, fructose, insulin resistance, glycaemic control.*

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LIST OF ABBREVIATIONS

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin converting enzyme
ALA	α-Lipoic acid
ALT	Alanine transaminase
AMPK	Activated protein kinase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
AUC	Area under curve
BG	Blood glucose
BSA	Bovine serum albumin
CAT	Catalase
ChREBP	Carbohydrate response element-binding protein
CRP	C-reactive protein
DHAP	Dihydroxyacetone phosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoassay
ESI	Electrospray ionization
ER	Endoplasmic Reticulum
FBG	Fasting blood glucose
FFA	Free fatty acid
FRAP	Fe^{3+} reducing activity

GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon- like peptide –1
GLUT-4	Glucose type 4
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HbA1c	Glycated haemoglobin
HDL-cholesterol	High-density lipoprotein cholesterol
HFD	High-fat-diet
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LC-MS	Liquid chromatography–mass spectrometry
LDL-cholesterol	Low-density lipoprotein cholesterol
LPO	Lipid peroxidation
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
NF-κB	Nuclear factor kappa B
NO	Nitric Oxide
OGTT	Oral glucose tolerance test
PI3K	Phosphoinositide-3-kinase
PKB/Akt	Protein Kinase B

PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
SANS	South African National Standard
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
SGLT2	Sodium glucose co-transporter 2
SPSS	Statistical Package for the Social Sciences
STZ	Streptozotocin
SREBP-1c	Sterol regulatory element-binding protein-1c
T2D	Type 2 diabetes
TBARS	Thiobarbituric acid reactive substances
TG	Triglycerides
TLR	Toll-like reeptor
TNF-α	Tumour necrosis factor- α
TZDs	Thiazolidinediones
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoproteins
WST-1	Water-soluble tetrazolium salt

RESEARCH OUTPUTS

Publications

Article(s) published or in preparation from this thesis

1. **Mapasa NP**, Ntsapi CM, Makhafola TJ, Mashele SS, Chukwuma CI*. Unravelling the ameliorative mechanisms of Ellagitannins from pomegranate peel on fructose-induced detrimental metabolic alterations in rats (***in preparation***).

Other co-authored article(s) published or in preparation

1. Izu GO, **Mapasa NP**, Nambooze J, Chukwuma MS, Njoya EM, Tabakam GT, Bonnet SL, Makhafola TJ, Mashele SS, Chukwuma CI*. (2024). Modulated glucose uptake in Chang cells and suppressed ROS production in RAW 264.7 macrophages. Antioxidants, 13(10), 1233. https://doi.org/10.3390/antiox1310133

Conference presentation(s)

1. **Mapasa NP**, Ntsapi CM, Chukwuma CI*. Ellagitannins from pomegranate peel ameliorates insulin resistance, pro-inflammatory signalling and oxidative stress linked to fructose-induced metabolic disorders in rats. The 58th Annual South African Society for Basic and Clinical Pharmacology Conference (SASBCP2025) held at 14 Palm Boulevard Umhlanga Ridge, Umhlanga Rocks, Durban from 14th to 16th September 2025 (***oral presentation***).

CHAPTER 1

INTRODUCTION

Diabetes mellitus is a complex metabolic disorder. It is an abnormal physiological condition that is indicated by the continual elevation of blood glucose levels (hyperglycaemia) (Banday *et al.*, 2020). Hyperglycaemia results from abnormalities in insulin secretion, and insulin action, or both. Diabetes is broadly categorised into two main types: type 1 diabetes (T1D) and type 2 diabetes (T2D). T2D is a key subject of this thesis, due to the high prevalence and strong link to behavioural risk factors. In South Africa, for example, the prevalence has almost tripled between 2010 and 2019 (Grundlingh *et al.*, 2022). T2D is the result of the interaction of multiple risk factors, including lifestyle factors, genetic factors, and medical factors (Crummett & Aslam, 2023).

Regardless of the lifestyle risk factors involved in the development of T2D, insulin resistance appears to be a common denominator in the pathophysiology of T2D. The pathophysiology of T2D is the malfunctioning of the feedback loop between insulin secretion and insulin action, disrupting glucose homeostasis, leading to abnormally high glucose levels (Röder *et al.*, 2016). When insulin action is impaired, the pancreas compensates by increasing insulin secretion to maintain normal glucose levels. However, if this compensatory mechanism fails due to beta-cell (β-cell) dysfunction, insulin production becomes insufficient, leading to persistent hyperglycaemia (Galica-Garca *et al.* 2020; Westman, 2021; Młynarska *et al.*, 2025). Insulin resistance is also central in the pathophysiology of metabolic syndrome, which is recognised as a key risk factor for T2D and cardiovascular disease (Nsiah *et al.*, 2015; Regufe *et al.*, 2020).

Metabolic syndrome is the dysregulation of metabolic processes, including insulin sensitivity, which increases the risk of developing metabolic and cardiovascular diseases, including T2D, atherogenic dyslipidaemia, hyperinsulinemia, central obesity, and hypertension. The pathophysiologic mechanisms underlying the development of metabolic syndrome involve several complex mechanisms, which are still being elucidated (Fahed *et al.*, 2022). According to some expert opinions, insulin resistance and central obesity are the key metabolic disorders that predispose an individual to metabolic syndrome and the risk of diseases, such as atherosclerosis and T2D (Regufe *et al.*, 2020).

Additionally, insulin remains a known regulatory hormone for lipid metabolism in adipose tissue. Thus, insulin resistance can lead to dysregulated lipid metabolism and disorders associated with metabolic syndrome and T2D. During insulin resistance, the lipolytic process

is impaired in fat tissues, causing the accumulation of circulating free fatty acids (FFA) (Raje *et al.*, 2020; Regufe *et al.*, 2020). This can alter insulin signalling in different tissues, which exacerbates insulin resistance in peripheral tissues. Insulin resistance triggers the release of pro-inflammatory cytokines in adipose tissue, which promotes a prothrombotic state (Ahmed *et al.*, 2021). Notably during metabolic syndrome, fat-induced oxidative stress can activate downstream dysregulation of pro-inflammatory responses (Khodabandehloo *et al.*, 2016; Fahed *et al.*, 2022).

Meanwhile, the pathophysiology of metabolic syndrome is complex, involving an intricate interplay of metabolic dysfunctions. The primary underlying factor is an imbalance of caloric intake and energy expenditure, compounded by a sedentary lifestyle and high-fat diet (Ahmed *et al.*, 2021). Central obesity, particularly visceral adiposity, is a key contributor, alongside insulin resistance and chronic inflammation (Ahmed *et al.*, 2021; Fahed *et al.*, 2021).

It has been observed that an unhealthy diet is a risk factor for obesity and metabolic syndrome. Documented reports have shown that consumption of diets high in saturated and trans fats, refined carbohydrates, including added sugars (sucrose and fructose), increases the risk of metabolic syndrome (Wong *et al.*, 2016; Rodríguez-Correa *et al.*, 2020). The overconsumption of fructose has been linked to insulin resistance, which contributes to several adverse health effects, including systemic inflammation, elevated uric acid, oxidative stress, increased triglyceride concentrations, and elevated blood pressure (Basciano *et al.*, 2005; Muriel *et al.*, 2021). The mortality and morbidity outcomes associated with metabolic disorders or metabolic syndrome have prompted the continuous search for different therapeutic approaches to combat this plague.

The management of metabolic syndrome comprises different therapeutic approaches, which include pharmacological and non-pharmacological approaches. Therapeutic approaches include the use of blood glucose-lowering agents such as the glucagon-like peptide-1 (GLP-1) receptor agonists, biguanides, thiazolidinediones, sulfonylureas, sodium-glucose co-transporter-2 (SGLT-2) inhibitors, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, and alpha-glucosidase inhibitors, as well as commercial drugs for obesity and cardiovascular diseases (Feingold, 2024). Despite the above-mentioned therapeutic approaches, lifestyle modifications/interventions, including diet, exercise, and supplements, remain the most effective approach for improving metabolic health and reducing the risk of metabolic syndrome.

Studies have shown that physical activity has a protective effect on a few biological pathways in the development of T2D. For instance, Sami *et al.* (2017) opine that physical activity increases insulin sensitivity by improving glucose tolerance *et al.*. Moreover, interventional studies have demonstrated that diets high in monounsaturated and/or complex carbohydrates can improve insulin sensitivity (Sami *et al.*, 2017; Yeh *et al.*, 2023). Additionally, nutritional supplements, including vitamins, minerals, and natural polyphenols, have also been linked to health benefits that could mitigate many risk factors relating to metabolic disorders. Nutritional supplements are often used by patients either as an alternative or in addition to their prescribed medications, with the intent to prevent or manage metabolic diseases such as T2D (Fong *et al.*, 2022; Raghuvanshi *et al.*, 2023; Martiniakova *et al.*, 2025). Furthermore, research evidence has shown that plant polyphenols have the potential to reduce the risk of metabolic and vascular diseases, as well as associated oxidative complications (Gasmi *et al.*, 2022; Iqbal *et al.*, 2023). Polyphenols are widely distributed across various plant sources, including fruits and vegetables (Bahadoran *et al.*,2013; Bertelli *et al.*, 2021; Rana *et al.*, 2022). They include diverse compounds that are classified into different classes, such as the nonflavonoids, tannins, and flavonoids, which are categorised based on the structure of their phenolic rings (Abbas *et al.*, 2016; Tijjani *et al.*, 2020).

Pomegranate is one of the few fruits with notable contents of hydrolysable tannins. Traditionally, the pomegranate has been used to treat several health problems, such as stomach aches and bacterial infections. Pharmacological studies have also shown the anti-inflammatory, antioxidant, cardio-protective, and anti-diabetic potentials of pomegranate (Holland *et al.*, 2009; Valero-Mendoza *et al.*, 2023; Ezeora *et al.*, 2024). A wealth of evidence has supported the cardiovascular and antioxidative health benefits of pomegranate fruit consumption, which has been majorly linked to the ellagitannins (punicalagins, ellagic acid, and related phytochemicals) and some proanthocyanidins present in the fruit. Likewise, advances in research have revealed that the peel of pomegranate is rich in these bioactive ellagitannins and some proanthocyanidins, making it a promising source of ellagitannins (Mashile *et al.*, 2024; Setlhodi *et al.*, 2024).

Ellagitannins are dietary bioactive polyphenols that belong to the class of hydrolysable tannins. They are metabolised by gut microbes into ellagic acid and urolithins, which are absorbed by the intestine (Banc *et al.*, 2023). Punicalagins are the main ellagitannins in pomegranate and are considered as the key bioactive ellagitannins influencing the metabolic and oxidative health benefits attributed to pomegranate (Heber *et al.*, 2007; Setlhodi *et al.*, 2024; Mashile *et al.*,

2024). For example, studies in both human and animals have documented the beneficial effects of punicalagin and ellagic acid on glycaemic control, lipids profiles, antioxidant status, inflammatory signalling and tissue damage in type 2 diabetic condition, hypertriglyceridemia and/or diet-induced metabolic disorders (Atrahimovich *et al.*, 2018; Jin *et al.*, 2020; Ghadimi *et al.*, 2021; Zhao *et al.*, 2021; Quirós-Fernández *et al.*, 2022). However, it remains elusive whether ellagitannins from pomegranate peel can mitigate or suppress the detrimental effects caused by high consumption of added sugars, such as sucrose and fructose.

Therefore, this study was done to investigate the effect of an ellagitannins-rich fraction obtained from pomegranate peel on some metabolic alterations in rats that are caused by high fructose consumption.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes

Diabetes Mellitus is a complex metabolic disorder; it is an abnormal physiological condition that is indicated by the continual increase of blood glucose levels, hyperglycaemia (Banday *et al.*, 2020). Hyperglycaemia is the result of insulin secretion and insulin action abnormalities. It can be either one or a combination of both. The abnormalities in insulin secretion and/or action manifest chronically and heterogeneously as disturbances in carbohydrate, fat, and protein metabolism (Banday *et al.*, 2020). These dysfunctions affect multiple organs and disrupt their normal functioning. The progression of diabetes is gradual and results from the adverse effects of hyperglycaemia and associated metabolic anomalies on the normal structure and functioning of the micro-vasculature and macro-vasculature, leading to complications in the nerves, eyes, heart, and kidneys (Ogurtsova *et al.*, 2022).

As mentioned in chapter 1, diabetes is classified into two main types, namely type 1 diabetes and type 2 diabetes. Type 1 diabetes is insulin-dependent diabetes and is known to be juvenile-onset diabetes. It is about 5-10% of diabetes cases. It is known to be an autoimmune disorder, as it involves T-cell-mediated destruction of the pancreatic beta-cells, resulting in insulin deficiency and subsequently leading to hyperglycaemia. Its pathogenesis is not fully understood, still it is found to be greatly influenced by both genetic and environmental factors. The rate of its development is rapid in most cases, with the presentation of severe hyperglycaemia, the destruction of the beta-cells can lead to diabetic ketoacidosis (Barnett, 2018; Banday *et al.*, 2020).

Type 2 diabetes (T2D) is non-insulin-dependent diabetes. It is about 90-95% of diabetes cases. It is known to be a lifestyle-induced disease, as it results from interactions between genetic, environmental, and behavioural risk factors (Galica-Garcia *et al.*, 2020). It is these risk factors that therefore affect the response to insulin and lead to the disruption of various pathways, causing insulin resistance and pancreatic beta-cell dysfunction. Due to the high prevalence and strong link with behavioural risk factors, T2D is a key subject of this thesis. In South Africa, for example, the T2D prevalence has almost tripled between 2010 and 2019 (Grundlingh *et al.*, 2022).

T2D is the result of the interaction of many risk factors, such as lifestyle factors, genetic and medical factors. Lifestyle factors include obesity and excess body fat percentage, especially around the abdominal area. The body fat percentage is considered due to the role of adipose tissue in promoting insulin resistance (Bellou, *et al.*, 2018; Ruze *et al.*, 2023). Physical inactivity is another lifestyle factor that increases insulin resistance. Without activity, the body becomes less responsive to insulin, contributing to excess weight and elevating blood pressure, cholesterol, and inflammation (Eaton & Eaton, 2017). Also, documented evidence has shown that an unhealthy diet, excess sugar, refined carbohydrates, high saturated and trans-fat, low fibre, and lack of nutrient-dense foods may lead to blood spikes, insulin resistance and unregulated blood sugar (Crummett & Aslam, 2023). Regardless of the lifestyle risk factor, insulin appears to be a common denominator in the pathophysiology of T2D.

2.2 Pathophysiology of T2D and the role of insulin resistance

The malfunctioning of the feedback loop between insulin secretion and insulin action disrupts glucose homeostasis, leading to abnormally high glucose levels. Under normal conditions, inulin secretion from the pancreatic β-cells is tightly regulated according to blood glucose concentration and insulin sensitivity in peripheral tissues such as muscle, liver and adipose tissues (Röder *et al.*, 2016). Central to insulin-mediated glucose homeostasis is the signalling of glucose storage as glycogen and glucose uptake in peripheral tissues for cellular metabolism, as shown in **Figure 2.1**.

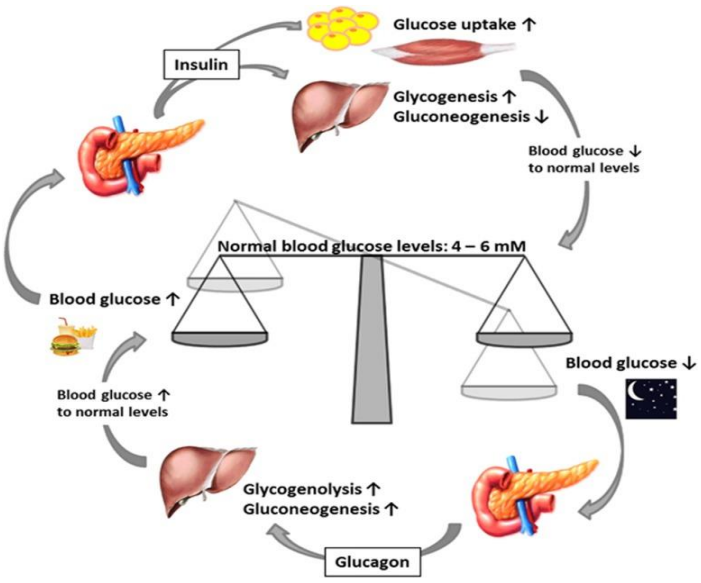


Figure 2.1: Hormone-mediated physiological glucose homeostasis (Röder *et al.*, 2016)

Insulin-mediated cellular glucose uptake signalling comprises a cascade of events initiated by the binding of insulin molecules to insulin receptors located on the cell membrane (**Figure 2.2**). The key signalling proteins of this cascade include insulin receptor substrate 1 (IRS-1), phosphoinositide-3-kinase (PI3K), protein kinase B (PKB or Akt), and glucose type 4 (GLUT-4). The binding of insulin to membrane insulin receptors signals a cascade of phosphorylative activation of these proteins, which eventually results in the translocation of GLUT-4 from the cytoplasm to the cell membrane, through which glucose enters the cells (Kim *et al.*, 2009).

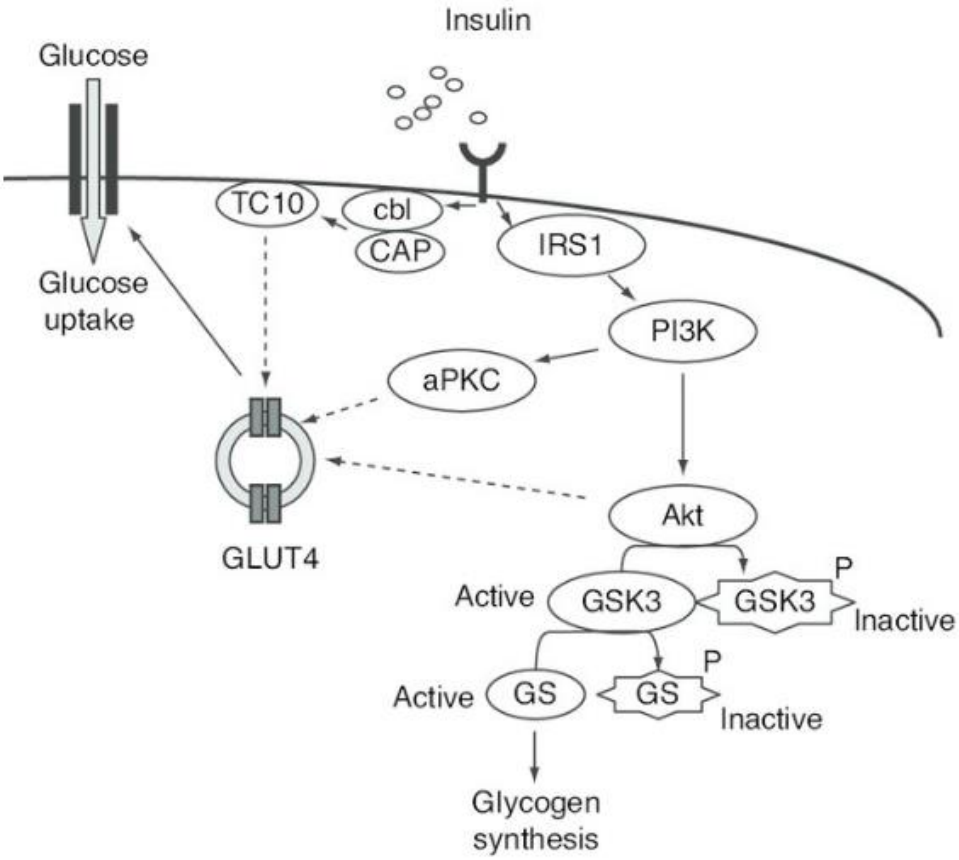


Figure 2.2: Insulin-mediated cellular glucose uptake signalling (Kim *et al.*, 2009)

When insulin action is impaired, the pancreas compensates by secreting more insulin to maintain normal glucose levels. However, if this compensatory mechanism fails due to beta-cell dysfunction, insulin production becomes insufficient, leading to persistent hyperglycaemia (Galica-Garca *et al.* 2020; Westman, 2021; Młynarska *et al.*, 2025).

As shown in **Figure 2.3**, defective insulin action primarily affects three key extra-pancreatic organs, namely skeletal muscles. adipose tissue, and the liver. In skeletal muscle, impaired insulin signalling reduces glucose uptake, leading to elevated blood glucose levels. In adipose tissue, dysfunctional insulin action promotes increased lipolysis, releasing excess free fatty

acids (FFAs) that exacerbate the impairment of insulin sensitivity. In the liver, insulin resistance results in excessive glucose production and reduced glycogen storage, exacerbating hyperglycaemia. The combined dysfunction of these organs contributes to a systemic insulin resistance, which leads to the development of T2D (Galica-Garca *et al.* 2020; Lu *et al.*, 2024).

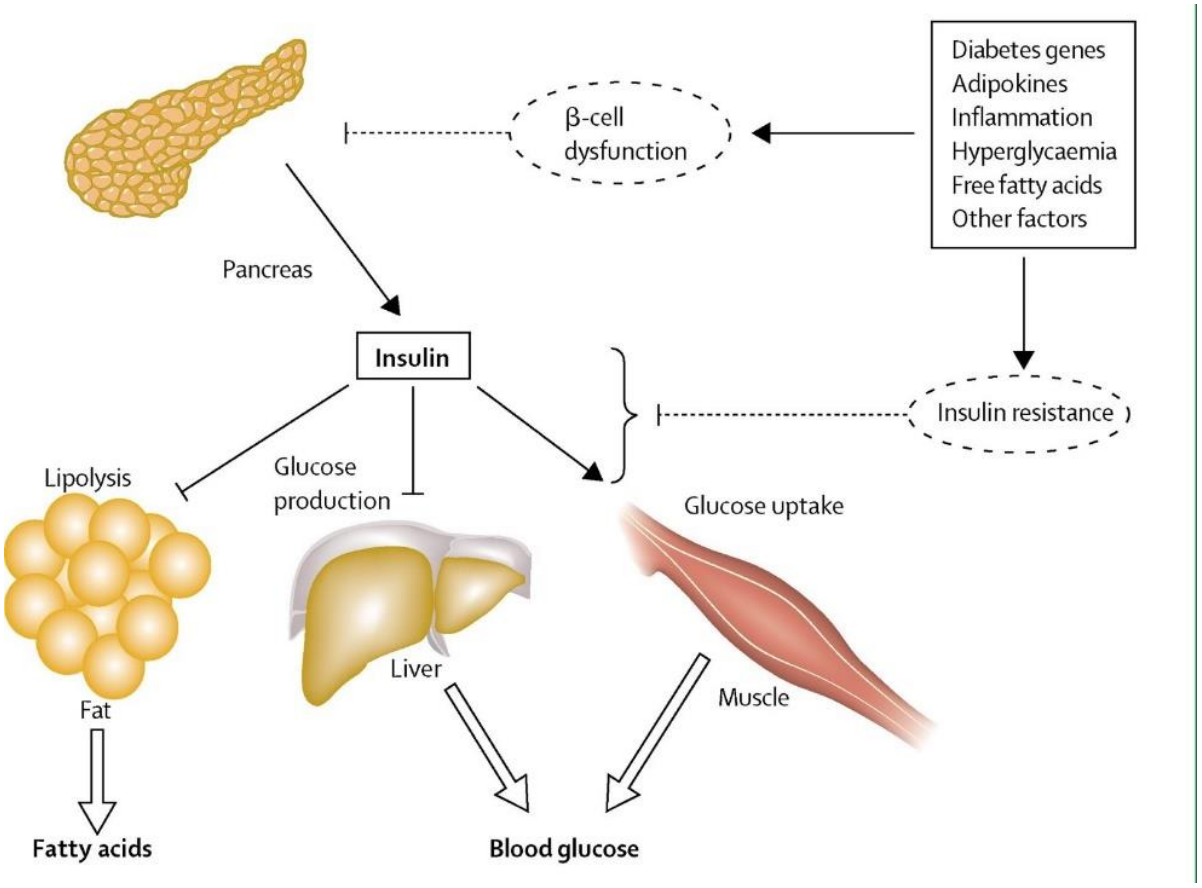


Figure 2.3: Pathologies of T2D (Stumvoll *et al.*, 2005)

Both processes can occur in early stages of developing T2D. The result of β-cell dysfunction is usually more severe than that of insulin resistance, and both are present; the hyperglycaemia is amplified, leading to the progression of T2D (Westman, 2021; Młynarska *et al.*, 2025). The mechanism leading to β-cell dysfunction is associated with the death of β-cells. Recent studies have suggested that the dysfunction of the β-cells in T2D may be due to more complex interactive network between environment factors and different molecular pathways linked to the functioning of β-cells (Galica-Garca *et al.*, 2020; Lu *et al.*, 2024; Młynarska *et al.*, 2025).

Excessive visceral adiposity and hyperlipidaemia often favour insulin resistance and chronic inflammation due to elevated free fatty acids release, which in the presence of hyperglycaemia, causes β-cell dysfunction. This can be linked to the induction of endoplasmic reticulum (ER) stress through the activation of the apoptotic signalling response (Galica-Garca *et al.*, 2020; Lu

et al., 2024; Młynarska *et al.*, 2025). Also, glucotoxicity, lipotoxicity, and glucolipotoxicity that occur during obesity can induce metabolic and oxidative stress, which can lead to β-cell damage. The disruption of islet integrity/organisation can occur, impairing the optimal communication between the cells of the pancreatic islets. This contributes to the poor regulation of insulin and glucagon release, worsening hyperglycaemia (Galica-Garca *et al.* 2020; Lu *et al.*, 2024; Młynarska *et al.*, 2025).

As consistently shown, insulin resistance remains central in the pathophysiology of T2D and related metabolic disorders. Hence, metabolic syndrome is recognised as a main risk factor for T2D, and cardiovascular disease (Nsiah *et al.*, 2015; Regufe *et al.*, 2020).

2.3 Metabolic syndrome, type 2 diabetes and the role of insulin resistance

Metabolic syndrome comprises a dysregulation of metabolic processes, including insulin sensitivity, which increases the risk of developing metabolic and cardiovascular diseases and disorders, such as T2D, atherogenic dyslipidaemia, hyperinsulinemia, central obesity, and hypertension (Fahed *et al.*, 2022). The pathophysiologic mechanisms underlying the development of metabolic syndrome involve several complex mechanisms that are still being elucidated. It is, however, the opinion of some scholars that the pathologies of the diseases associated with metabolic syndrome could be interconnected and perhaps are common to the same pathogenic process (Fahed *et al.*, 2022; Patial *et al.*, 2024) as shown in **Figure 2.4**. In fact, metabolic syndrome shares similar risk factors with T2D, which include genetic, lifestyle and environmental factors (Regufe *et al.*, 2020).

Metabolic syndrome may occur before, or coexist with T2D, which has been implicated in the high occurrence of cardiovascular diseases and associated mortalities in diabetic patients (Asghar *et al.*, 2023). Excess abdominal fat (abdominal circumference exceeding 102 cm and 88 cm in men and women, respectively), abnormal levels of blood lipids (triglycerides > 150 mg/dL; HDL-cholesterol ≤ 40 mg/dL and 50 mg/dL in men and women, respectively), high blood pressure (≥ 35/85 mm Hg), and fasting blood glucose (≥100 mg/dL) are the classical clinical diagnostic indicators of metabolic syndrome, and they have been indicated as clinical biomarkers that increases the risk of T2D, and cardiovascular diseases (Regufe *et al.*, 2020).

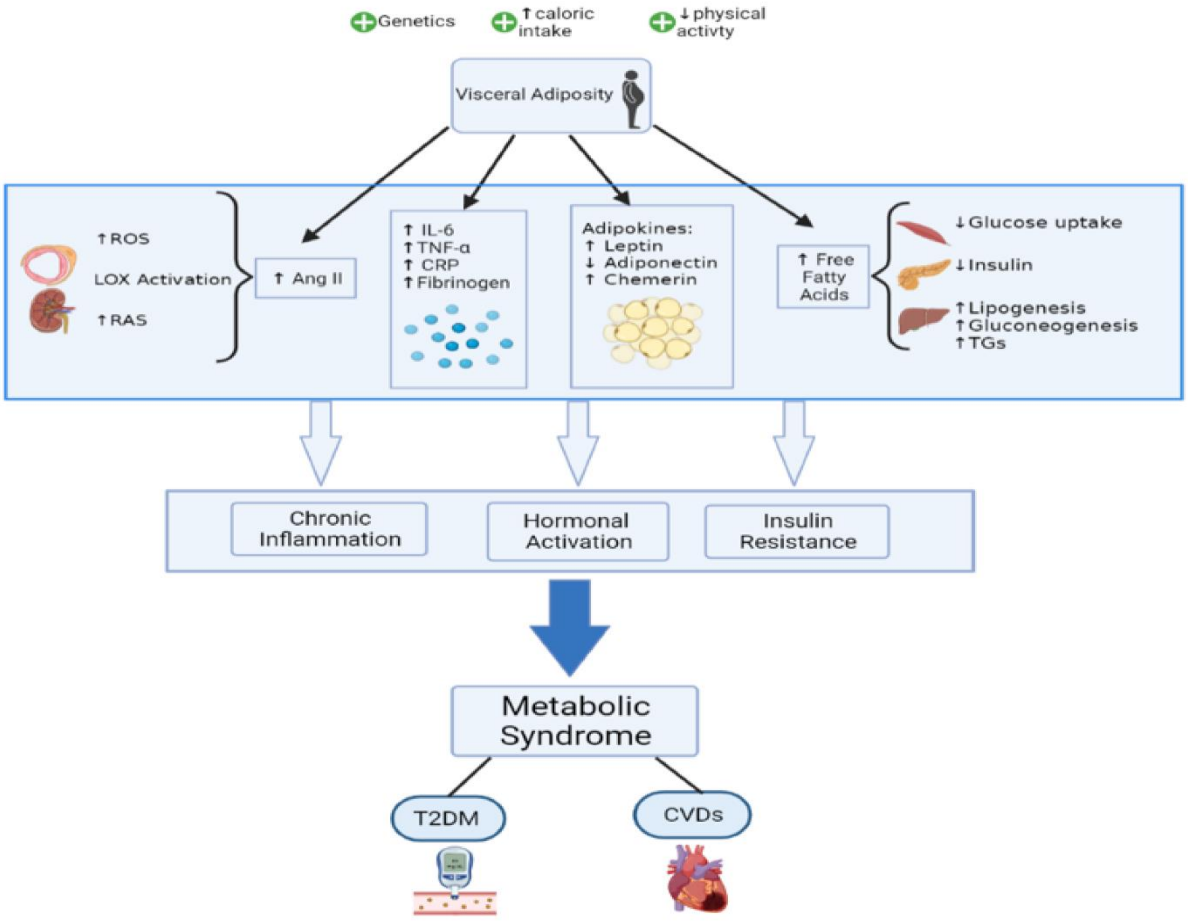


Figure 2.4: Highlighted mechanisms underlying the pathophysiology of metabolic syndrome (Fahed *et al.*, 2022)

According to expert opinions, insulin resistance and central obesity are the key metabolic disorders that predispose an individual to metabolic syndrome and the risk of diseases such as atherosclerosis and T2D (Regufe *et al.*, 2020). Evidence has shown that lipolysis in abdominal (visceral) adipose tissue is a notable influential contributor to insulin resistance than lipolysis in subcutaneous fat. This is because, through the splanchnic circulation, lipolytic action in abdominal adipose tissues directly releases free fatty acids (FFAs) into the liver, promoting insulin resistance and impairing insulin-mediated nutrient metabolism (Patel & Abate, 2013).

2.3.1 Insulin resistance in lipid metabolism

Insulin is a known regulatory hormone for lipid metabolism in adipose tissue. Thus, insulin resistance can result in dysregulated lipid metabolism and disorders associated with metabolic syndrome and T2D. During insulin resistance, the lipolytic process is impaired in fat tissues,

causing the accumulation of circulating FFAs (Raje *et al.*, 2020; Regufe *et al.*, 2020). This can alter insulin signalling in different tissues, which exacerbates insulin resistance in peripheral tissues (**Figure 2.5**). The insulin-mediated inhibition of lipolysis in adipose tissues is 10-fold lesser in individuals having metabolic syndrome than in healthy individuals (Kerr *et al.*, 2024),thus influencing the status of their medical conditions.

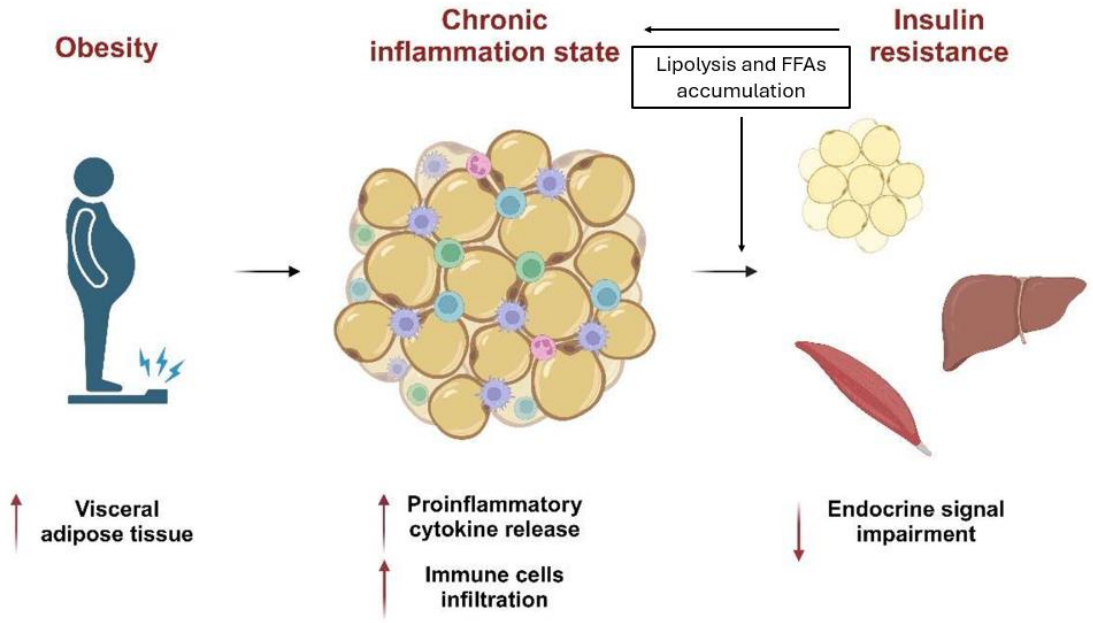


Figure 2.5: Role of insulin resistance and adiposity in metabolic disorders (Secchiero *et al.*, 2024)

Furthermore, the accumulation of FFAs can adversely affect key insulin signalling steps in muscle, including IRS-1-mediated PI3K activity (Fahed *et al.*, 2022). This disruption impairs GLUT-4 translocation to the cell membrane and subsequently reduces cellular glucose uptake. Additionally, in hepatocytes, the accumulation of FFAs could lead to increased glucose (gluconeogenesis) and lipid synthesis (lipogenesis) . Combined with enhanced lipolysis, these effects contribute to a hyperinsulinemic state, in which compensatory insulin secretion attempts to maintain glucose homeostasis despite insulin resistance (Fahed *et al.*, 2022).

In terms of cardiovascular consequences, FFAs accumulation associated with insulin resistance contributes to cardiovascular impairments. Also, elevated FFAs enhance the synthesis of cholesterol esters and triglycerides, thereby promoting the overproduction of triglyceride-rich low-density lipoproteins (VLDLs). In this condition, cholesterol ester transfer protein facilitates the exchange of triglycerides from VLDLs to high-density lipoproteins (HDLs),

leading to HDL depletion. This reduction in protective HDL . constitutes a key pathological factors in atherogenic dyslipidaemia (Fahed *et al*, 2022).

2.3.2 The role of inflammatory responses and oxidative stress

Pro-inflammatory cytokines release has been documented as a possible pathophysiological mechanism through which insulin resistance increases the risk of developing cardiovascular diseases (Ahmed *et al.*, 2021). Insulin resistance triggers pro-inflammatory cytokines released in adipose tissue, which promotes a prothrombotic state (Ahmed *et al.*, 2021). During metabolic syndrome, fat-induced oxidative stress can activate downstream dysregulation of pro-inflammatory responses. Studies have shown that there is increased expression of pro-inflammatory cytokines and/or markers, such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukins-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-alpha (TNF-α), high sensitivity C-reactive protein (CRP) and reduced expression of anti-inflammatory cytokines and/or markers, such as interleukins-10 (IL-10) and adiponectin (Khodabandehloo *et al.*, 2016; Fahed *et al.*, 2022) (**Figure 2.6**).

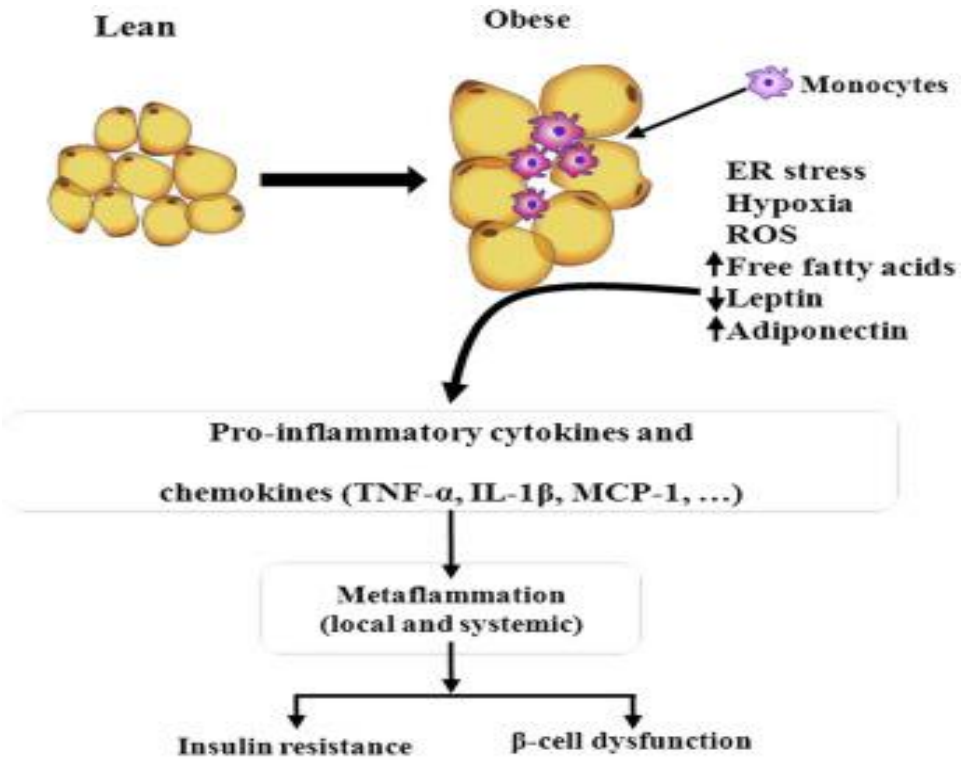


Figure 2.6: The adiposity, oxidative stress and pro-inflammatory responses in metabolic disorders (Khodabandehloo *et al.*, 2016)

Through the diversion of FFAs from abdominal adipose tissues to the liver via the portal circulation, insulin resistance in adipose tissues worsens metabolic dysfunction (Ahmed *et al.*, 2021; Fahed *et al.*, 2022). The susceptibility of visceral adipose tissues to insulin resistance also makes this process highly likely in individuals with excess abdominal fat and at risk of metabolic syndrome. This adipose tissue-liver metabolic dysfunction results in the release of pro-inflammatory cytokines and other markers (Ahmed *et al.*, 2021; Fahed *et al.*, 2022).

During metabolic disorders, there is notable infiltration of macrophages into visceral adipocytes. Inflammatory cytokines and chemokines are produced in visceral adipose tissue from these localised macrophages, depending on the mass of adipose tissue and level of insulin resistance, which contributes to systemic inflammation and further exacerbates insulin resistance in adipose tissue and hepatocytes (Hachiya *et al.*, 2022; Gkrinia and Belančić, 2025). Other immune cells, including T cells and neutrophils, also potentiate inflammation in insulin-resistant cells of peripheral tissues (Hachiya *et al.*, 2022). Additionally, insulin resistance-mediated increases in lipolysis and FFAs accumulation can directly modulate the inflammatory pathways, particularly through immune receptors like Toll-like receptors (Fahed *et al.*, 2022).

Visceral adiposity-induced inflammation is driven by a dysregulation in the activation of pro-inflammatory signalling pathways, especially the c-Jun-N-terminal kinase (JNK) and nuclear factor kappa B (NF-κB) (Gkrinia & Belančić, 2025). Nuclear factor kappa B is a transcription factor that is activated by reactive oxygen species (ROS), among other stimuli like cytokines and FFAs accumulation, suggesting that oxidative stress is associated with insulin resistance-mediated inflammatory responses in peripheral tissues (Masenga *et al.*, 2023). Oxidative stress has been linked to mitochondrial dysfunction, which can influence inflammatory responses due to the deleterious effects of ROS on cellular components (Masenga *et al.*, 2023). Oxidative stress is considered an upstream occurrence that potentiates inflammation, because it stimulates the monocytes and macrophages activation (Yaribeygi *et al.*, 2020).

Meanwhile, the pathophysiology of metabolic syndrome is complex, involving an intricate interplay of metabolic dysfunctions. The primary underlying factor is an imbalance of caloric intake and energy expenditure, compounded by a sedentary lifestyle and high-fat diet (Ahmed *et al.*, 2021). Central obesity, particularly visceral adiposity, is a key contributor, alongside insulin resistance and chronic inflammation (Ahmed *et al.*, 2021; Fahed *et al.*, 2021).

2.4 Diet-induced metabolic disorders

It is well known that an unhealthy diet is a risk factor for obesity and metabolic syndrome. Evidently, the consumption of diets high in saturated and trans fats and refined carbohydrates, including added sugars (sucrose and fructose), increases the risk of metabolic syndrome (Wong *et al.*, 2016; Rodríguez-Correa *et al.*, 2020).

2.4.1 Metabolic disorder induced by a high-fat diet

Fats are essential macronutrients, and they are calorically denser than other macronutrients. Fats consist of esters of three fatty acid chains and glycerol. (Wong *et al.*, 2016). Lipid metabolism is initiated through the process of lipolysis. The breakdown of fats releases many glycerol and fatty acids, which are freely diffused into the circulation. Once the FFAs are in the plasma, they are transported to the liver, where they are a major precursor used to produce VLDL-triglycerides. More than half of the released FFAs enter the process of lipogenesis and are re-esterified (Wong *et al.*, 2016; Edwards & Mohiuddin., 2023).

Pertaining to VLDL, it assists in the circulation of fats in a water-based blood solution. The increased formation of VLDL by the liver is a compensatory response to excess FFAs and increased synthesis of triglycerides. The excess lipogenesis contributes to visceral fat accumulation. Visceral adiposity, in turn, exhibits enhanced lipolytic activity, releasing FFAs into the portal circulation. These FFAs are taken up by the liver, re-esterified into triglycerides, and contribute to the formation of VLDL (Unger & Scherer, 2010; Wong *et al.*, 2016). Elevated level of VLDL cholesterol is a risk factor for obesity, dyslipidemia, arterial accumulation of cholesterol and cardiovascular diseases, of which insulin resistance is a major culprit. A high-fat diet often drives the sequence of events through the development of insulin resistance (Unger & Scherer, 2010; Wong *et al.*, 2016).

Based on their findings, Rodríguez-Correa *et al.* (2020), Wong *et al.* (2016), and Fraulob *et al.* (2010) have reported that high dietary fat increased leptin, blood glucose, weight gain, fat mass, cholesterol, triglycerides, unesterified cholesterol, and LDL-cholesterol in animals, causing insulin resistance.

2.4.2 Metabolic disorder induced by a high-carbohydrate-enriched diet

Carbohydrates are classified into simple and complex forms. Carbohydrates are essential nutrients that act as the body's primary source of energy. This source of energy is called short-term fuel and is readily metabolised compared to fats, aiding in the control of blood glucose and the metabolism of insulin. In the presence of a sedentary lifestyle, a high-carbohydrate diet contributes to increased energy storage, leading to overweight and eventually obesity (Wong *et al.*, 2016; Holesh *et al.*, 2023).

Carbohydrates are broken down into glucose molecules, which are absorbed in the small intestine into circulation. The glucose molecules are, then, transported via the portal vein to the liver, where they are stored as glycogen. Glucose is also taken up by actively respiring cells through insulin signalling to undergo glycolysis and energy production. However, prolonged consumption of excess carbohydrate signals a fed-state, promoting insulin-mediated anabolic processes through various pathways. One of such pathways is the conversion of excess glucose as fats in hepatic adipocytes, a process often referred to as *de novo* lipogenesis. This can promote visceral adiposity, which is a risk factor for obesity, insulin resistance, and dyslipidaemia (Wong *et al.*, 2016; Yoo *et al.*, 2024).

Moreover, evidence shows that high-carbohydrate diets intake results in increased *de novo* lipogenesis in the liver has been documented (Santos & Penha-Silva, 2025). Long-term intake of a high-carbohydrate diet has been reported to cause greater hepatic triacylglycerol accumulation than a high-fat diet in experimental animals (Karasawa *et al.*, 2023)

2.4.3 Metabolic disorder induced by a sucrose-enriched diet

Sucrose is a disaccharide that is composed of glucose and fructose. It is commonly used to make confectionaries. Once sucrose is consumed, it will be broken down by the sucrase enzyme into glucose and fructose. Each of these monosaccharides will then follow its respective metabolic pathway (Gibson *et al.* 2013; Wong *et al.* 2016).

Fructose is known to be a more efficient substrate for fatty acid synthesis in the liver, compared to glucose. Phosphofructokinase, a key regulatory enzyme in glycolysis, negatively regulates glucose metabolism. This inhibition of glucose uptake causes the steady entry of fructose into the glycolytic pathway (Wong *et al.* 2016; Chaudhry & Varacallo. 2023). Therefore, an

oversupply of fructose will be more readily converted to fat, suggesting that fructose is one of the main culprits of hepatic insulin resistance (Wong *et al.*, 2016; Taskinen *et al.*, 2019)

A study conducted using rats showed that 4 weeks of administration of a 30% sucrose solution in place of drinking water significantly increased detrimental blood lipids (triglycerides and LDL-cholesterol) (Carvajal-Zarrabal *et al.*, 2014).

2.4.4 Fructose-induced metabolic alterations

In terms of dietary needs, there is no biological need for fructose. (Basciano *et al.*, 2005; Wong *et al.*, 2016; Taskinen *et al.*, 2019). Fructose metabolism primarily occurs in the liver, which plays a central role in maintaining the whole body's energy homeostasis. The liver plays a crucial role in regulating various metabolic processes, including those of carbohydrates, lipids, proteins, and xenobiotics. This organ is also good for detoxification. These regulatory functions are carried out through key pathways, including gluconeogenesis, glycogenolysis, glycogenesis, lipogenesis, cholesterol synthesis, and the synthesis of coagulation factors (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Muriel *et al.*, 2021; Meshkani & Adeli, 2009).

Once fructose reaches the liver, it is metabolised through its distinct pathway. Fructose is converted into fructose-1-phosphate by the fructokinase enzyme. Then, fructose-1-phosphate is cleaved into dihydroxyacetone phosphate (DHAP) and glyceraldehyde by the aldolase enzyme, bypassing the regulatory step involving phosphofructokinase in glycolysis. With fructose metabolism bypassing the regulatory step, it leads to unregulated glycolytic intermediates, which are precursors of hepatic lipids, such as triglycerides (Basciano *et al.*, 2005; Wong *et al.*, 2016; Taskinen *et al.*, 2019). The above information is further illustrated in **Figure 2.7**. Moreover, fructose is also known to inhibit lipid oxidation in the liver, which favours the synthesis of VLDL-triglycerides, and re-esterification of fatty acids.

The overconsumption of fructose has been linked to insulin resistance, which contributes to several adverse health effects, including systemic inflammation, elevated uric acid, oxidative stress, increased triglyceride concentrations, and elevated blood pressure. (Basciano *et al.*, 2005; Muriel *et al.*, 2021). Thirunavukkarasu et al. studied rats fed a diet enriched with 60% fructose and found that it led to increased glucose intolerance and blood pressure, along with decreased insulin sensitivity. (Thirunavukkarasu *et al.*, 2016). The cause of hepatic insulin resistance is from multiple molecular mechanisms that are interlinked; it is the disruption of

the normal insulin signalling and glucose metabolism (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009).

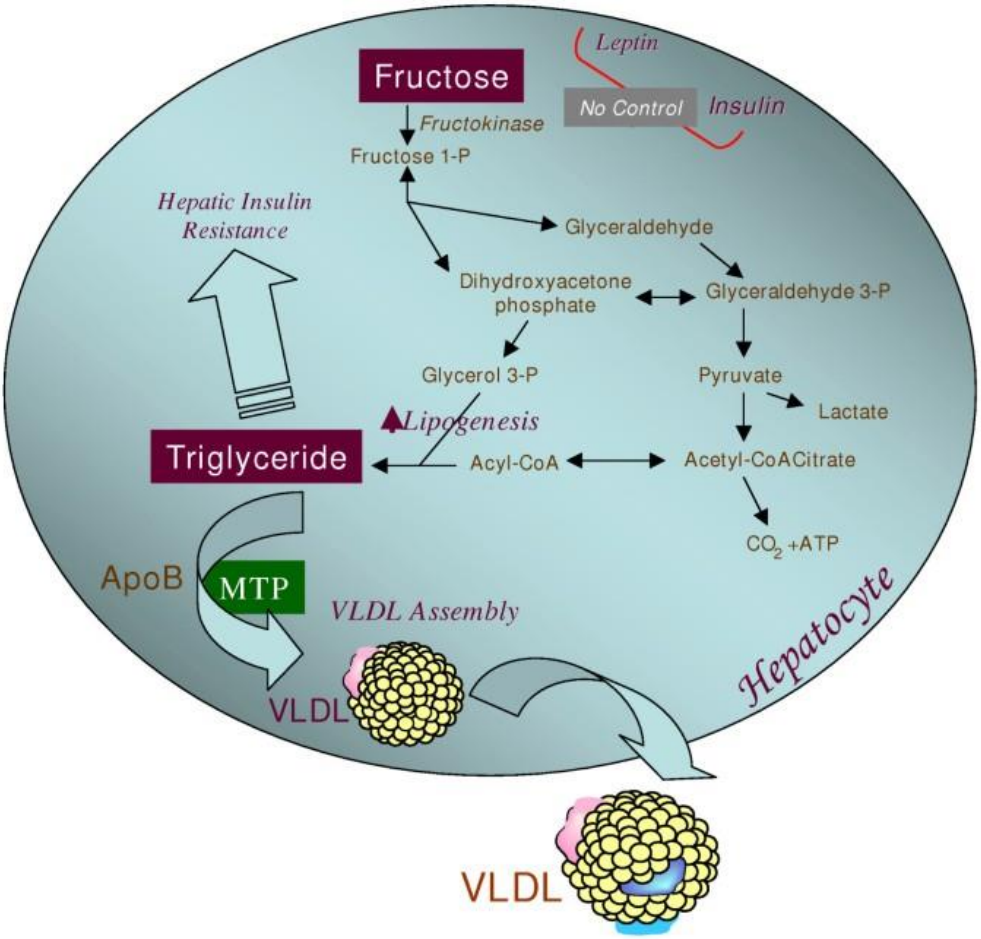


Figure 2.7: Hepatic fructose metabolism, insulin resistance dyslipidaemia (Basciano *et al.*, 2005)

One of the most critical pathways involved in the hepatic insulin signalling is the PI3K-Akt pathway, as it is an insulin-dependent signalling cascade involving phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt). Upon insulin binding to its receptor, this pathway is activated and plays an important role in glucose metabolism by promoting glycogen synthesis and enhancing glucose uptake in liver cells (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009). However, when there is metabolic stress, the pathway may become impaired, leading to a reduction in glycogen storage and an increase in hepatic glucose production. Such that these changes contribute to hyperglycaemia and the development of insulin resistance. The disruption of the PI3k-Akt pathway compromises the liver's ability to

respond effectively to insulin, making it a central molecular pathway in the progression of metabolic dysfunction (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009).

Excessive fructose consumption could lead to lipid accumulation in the liver, which exacerbates insulin resistance. Excessive free fatty acids and triglycerides build up in the liver, leading to hepatic steatosis. This build-up of lipids in the liver leads to interference with insulin signalling pathways, disrupting the normal function of insulin. This disruption activates stress and inflammatory responses within the liver, which further impair glucose metabolism. As the regulation of glucose becomes more dysfunctional, the insulin resistance worsens. This, in turn, promotes more lipid accumulation in the liver, and creates a vicious cycle, whereby fat build-up and metabolic dysfunction continuously reinforce one another (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009). Fructose consumption is also known to inhibit lipid oxidation in the liver, favouring the synthesis of VLDL-triglycerides and re-esterification of fatty acids, which exacerbates insulin resistance (Basciano *et al.*, 2005) (**Figure 2.7**).

Chronic inflammation also plays a critical role in hepatic insulin resistance. Chronic inflammation triggers the release of pro-inflammatory cytokines, such as tumour necrosis factor-alpha and interleukin-6. The release of these cytokines activates cellular stress pathways, which interfere with insulin. This results in the disruption of the normal function of insulin receptors, and intracellular signalling processes are disrupted. The interference impairs the liver's ability to regulate glucose effectively. Over time, chronic inflammation also increases oxidative stress and disturbs normal lipid metabolism and further worsening the insulin resistance in the liver (Basciano *et al.*, 2005; Meshkani &Adeli, 2009).

Furthermore, a fructose-enriched diet causes metabolic stress that disrupts normal cellular function of the liver. The stress impairs the activity of the endoplasmic reticulum (ER), an organelle that is responsible for protein folding and lipid metabolism. Such disruption leads in the accumulation of misfolded proteins that trigger ER stress and activate stress pathways that interfere with insulin signalling (Kelley *et al.*, 2004; Basciano *et al.*, 2005). Meanwhile, metabolic stress leads to oxidative stress, which is a condition whereby there is an in between reactive oxygen species and the body's antioxidant defences. This imbalance causes cellular damage, promotes lipid peroxidation, and disrupts insulin action. Over time, those anomalies create a harmful cycle of cellular stress and metabolic imbalance that leads to the progression of liver-related metabolic diseases (Kelley *et al.*, 2004; Basciano *et al.*, 2005). Additionally, the combination of endoplasmic reticulum and oxidative stress directly contributes to hepatic

insulin resistance and worsens metabolic dysfunction (Kelley *et al.*, 2004; Basciano *et al.*, 2005).

Another significant pathway contributing to hepatic insulin resistance is the JNK/NF-κB pathway, which becomes activated in response to chronic inflammation. Inflammatory signals stimulate the activation of c-Jun N-terminal kinase (JNK) and nuclear factor kappa B, which are molecules involved in cellular stress and immune responses. Once activated, the pathways interfere with insulin signalling by JNK-dependent serine phosphorylation of the insulin receptor substrate proteins (IRS-1 and IRS-2) and obstructing tyrosine phosphorylation (Regnault *et al.*, 2013). The chemical modification that occurred in IRS proteins reduces their ability to transmit insulin signals effectively within the liver cells (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009).

The activation of SREBP-1c and ChREBP pathway also plays a crucial role in the development of hepatic insulin resistance. Under conditions of high fructose intake, the liver activates two important transcription factors, namely sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP). These transcription factors stimulate hepatic lipogenesis, the process by which the liver synthesises fatty acids and triglycerides from excess nutrients (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009). When SREBP-1c and ChREBP are overactivated, they drive excessive lipid production, resulting in the accumulation of triglycerides in liver cells. The lipid overload disrupts insulin signalling, which leads to the development of hepatic steatosis (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009). This condition further promotes metabolic dysfunction, leading to a vicious feedback loop, where increased lipid buildup continues to impair insulin sensitivity, reinforcing the cycle of insulin resistance (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009).

The hexosamine biosynthetic pathway is another mechanism for fructose-induced hepatic insulin resistance. In this pathway, a small portion of glucose entering the cell is diverted from glycolysis and converted into hexosamine intermediates. These intermediates are used in a process called O-linked glycosylation, which adds sugar molecules to various proteins, including insulin-signalling molecules. As there is excessive glucose flux through the hexosamine biosynthetic pathway, it leads to increased glycosylation of key insulin signalling proteins, such as IRS proteins. This post-translational modification disrupts normal insulin receptor function, impairing the intracellular signalling cascade that promotes glucose uptake

and metabolism. The result is the development of hepatic resistance, making the liver less responsive to insulin (Kelley *et al.*, 2004; Basciano *et al.*, 2005; Meshkani & Adeli, 2009).

Essentially, the mortality and morbidity outcomes associated with metabolic disorders or metabolic syndrome have prompted the continuous search for different therapeutic approaches to combat this plague.

2.5 Management of metabolic disorders or metabolic syndrome

The management of metabolic syndrome comprises different therapeutic approaches, which includes pharmacological and non-pharmacological approaches.

2.5.1 Therapeutic targets

Several approaches have been used in managing metabolic syndrome. For individuals with T2D, impaired glucose tolerance, and persistent hyperglycaemia, commercial blood glucose-lowering agents are usually the clinically recommended option to manage T2D-related metabolic disorders. The blood glucose-lowering agents include the glucagon-like peptide-1 (GLP-1) receptor agonists, biguanides, thiazolidinediones, sulfonylureas, sodium-glucose co-transporter-2 (SGLT-2) inhibitors, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, and alpha-glucosidase inhibitors (Feingold, 2024).

Additionally, in the presence of obesity, cardiovascular diseases, and other commercially available drugs, such as antihypertensive drugs (angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, angiotensin receptor-neprilysin inhibitors, β-lockers, etc.), cardioprotective drugs (statins, antiplatelet agents, anticoagulants, antiarrhythmics, etc.), and anti-obesogenic agents may be used in combination with blood glucose-lowering agents (Cong *et al.*, 2020; Tchang *et al.*, 2024).

Some approaches have targeted inflammatory signalling. It is believed that drugs targeting inflammatory pathways, such as nuclear factor kappa B and c-Jun-N-terminal kinase pathways, could potentiate beneficial effects on metabolic syndrome (Welty *et al.*, 2016; Wang *et al.*, 2023). Other experts are of the opinion that the immune cells regulation could be another therapeutic target. Specifically, it is believed that reducing the infiltration of immune cells into adipocytes may be of benefit in managing metabolic syndrome (Varra *et al.*, 2024).

Furthermore, the role of antioxidant therapies cannot be overemphasised. It is well known that oxidative stress is implicated in the pathophysiology of metabolic syndrome and the associated pro-inflammatory disorders (Masenga *et al.*, 2023). Hence, antioxidant supplements can help to improve mitochondrial function and reduce oxidative stress-mediated inflammation in metabolic syndrome (Welty *et al.*, 2016; Fahed *et al.*, 2022).

Despite the above-mentioned therapeutic approaches, lifestyle modifications/interventions, including diet, exercise, and supplements, remain the most effective approach for improving metabolic health.

2.5.2 Exercise

Physical inactivity is known to increase insulin resistance. This lack of activity makes the body less responsive to insulin and leads to impaired glucose uptake, which in turn contributes to excess weight gain and elevates risk for the development of T2D and cardiovascular disease (Rana *et al.* 2007, Galica-Garca *et al.* 2020).

It has been reported that physical activity has a protective effect on a few biological pathways in the development of T2D (Sami *et al.* 2017). Physical activity increases insulin sensitivity by improving glucose tolerance. It also stimulates the skeletal muscle glucose uptake into the cells, thereby delaying the progression of T2D. Lastly, it reduces intra-abdominal fat. Visceral fat is strongly linked to insulin resistance. Regular physical activity has been shown to reduce this fat depot, lowering associated metabolic risks (Sami *et al.* 2017).

2.5.3 Diet and nutrition

The consumption of diets high in fat and refined carbohydrates has been identified as a significant risk factor for dyslipidemia, obesity, insulin resistance, and heart disease. There are many cross-sectional studies and meta-analyses that have reported strong associations between unhealthy diets increasing the risk of T2D (Zhao *et al.* 2022). There is a well-established connection between dietary intake and obesity, not only in terms of the number of calories but also the quality and composition of food consumed. Moreover, interventional studies have demonstrated that diets high in monounsaturated and/or high in complex carbohydrates can improve insulin sensitivity (Sami *et al.*, 2017; Yeh *et al.*, 2023). Improving dietary habits is

therefore a key strategy in managing T2D. Nevertheless, due to individual variability, there is no one-size-fits all diet for diabetic individual. As such, a personalised dietary planning is recommended to ensure optimal management and control of the disease (Sami *et al.*, 2017; Yeh *et al.*, 2023). Nonetheless, to reduce the risk of metabolic syndrome and T2D, it is recommended to limit glyceamic carbohydrates and consume more plant-based dietary fibres, fruits and vegetables (Wang *et al.*, 2015; Gerontiti *et al.*, 2024; Nedzingahe and Mbhenyane, 2024). Additionally, nutritional supplements, including vitamins, minerals and natural polyphenols have also been linked to health benefits that could mitigate the above-mentioned risks (Fong *et al.*, 2022; Raghuvanshi *et al.*, 2023; Martiniakova *et al.*, 2025).

2.5.4 Supplements

Nutritional supplements are often used by patients either as an alternative or in addition to their prescribed medications, with the intent to prevent or manage metabolic diseases such as T2D (Fong *et al.*, 2022). Various vitamins and compounds have been studied concerning their effects on diabetes, including inositols, vitamin D, niacin, nutraceutical (plant extracts, herbs, and essential oils), resveratrol, nicotinamide (B3), omega-3 polyunsaturated fatty acids, chromium, magnesium, fenugreek, curcumin extract, α-Lipoic acid (ALA), bitter melon, berberine, cassia cinnamon, flaxseed, ginseng, and gymnema (Valdés-Ramos *et al.*, 2015; Yeung *et al.*, 2018; Cross *et al.*, 2021; Petroni *et al.*, 2021).

However, the efficacy, safety, and potential for drug-supplement interactions remain critical considerations when evaluating the use of these products. While some supplements have shown some potentials in clinical studies, others have failed to demonstrate significant benefits. Given these uncertainties, lifestyle modifications, such as eating a healthy diet and exercising regularly, remain the most reliable and evidence-based strategy for the prevention and management of T2D (Valdés-Ramos *et al.*, 2015; Yeung *et al.*, 2018; Cross *et al.*, 2021; Petroni *et al.*, 2021). In spite of the above-mentioned limitations in the use of supplements, research evidence has shown that plant polyphenols have the potential to reduce the risk of metabolic and vascular diseases, as well as the associated oxidative complications (Gasmi *et al.*, 2022; Iqbal *et al.*, 2023)

2.6 Polyphenols

Polyphenols are micronutrients produced by plants as secondary plant metabolites. They serve multiple roles, including providing structural function, attracting pollinators, and protecting plants against infections, and ultraviolet radiation. These natural phytochemicals are widely distributed across various plant sources, particularly in fruits and vegetables (Bahadoran *et al.*,2013; Bertelli *et al.*, 2021; Rana *et al.*, 2022). Research has shown that consumption of diets rich in polyphenols exerts bioactive effects that can modulate inflammation and oxidative stress, as well as. improve blood pressure, lipid profile, systemic inflammation, and insulin resistance (Pandey & Rizvi, 2009; Bertelli *et al.*, 2021; Rana *et al.*, 2022). Polyphenols are structurally diverse compounds that are broadly classified into three groups: nonflavonoids, tannins, and flavonoids, depending on the structure of their phenolic rings. Each of these major classes have subclasses (**Figure 2.8**) (Abbas *et al.*, 2016; Tijjani *et al.*, 2020).

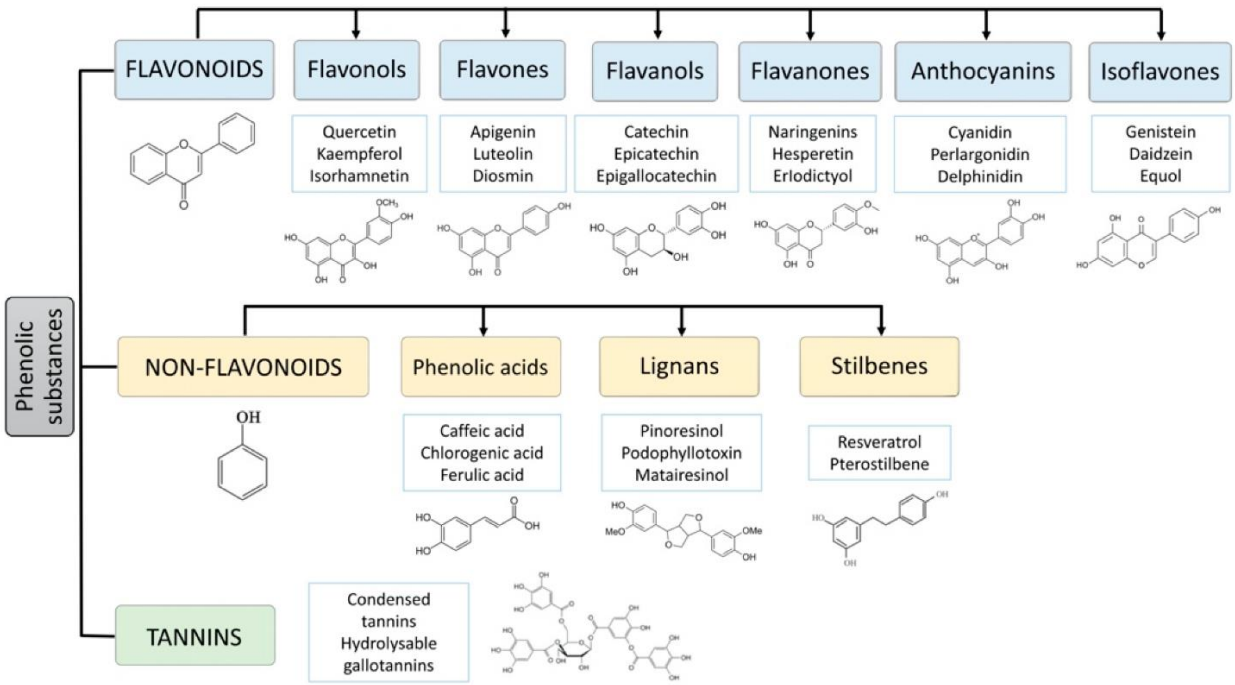


Figure 2.8: Major categories of plant-based polyphenols (Serra *et al.*, 2021)

Non-flavonoids are stilbenes, lignans, and phenolic acids. Stilbenes are a polyphenol group that has been researched as it is potent in their biological effects. It has been discovered that its consumption is associated with a lower risk of obesity, hypertension, diabetes, and reduced mortality overall (Chong *et al.*, 2009; Tresserra-Rimbau *et al.*, 2014; Miranda *et al.*, 2016; Sirerol *et al.*, 2016; Grosso *et al.*, 2017; Serra *et al.*, 2021). Lignans, also present in many plants, have been shown to have antihypertensive, anticancer, antioxidant, and antiviral effects

(Gorzkiewicz *et al.*, 2021; Deka *et al.*, 2022; Mukhija *et al.*, 2022; Yu *et al.*, 2022; Jang *et al.*, 2022; Serra *et al.*, 2021). Phenolic acids are a class of polyphenols that are known for their natural antioxidant properties, and they play crucial roles in plant growth, defense, and development, and they are also serve as precursors for other bioactive molecules (Kumar & Goel, 2019).

Meanwhile, flavonoids are common in many plants, including fruits and vegetables. They are categorised into subclasses: flavones, flavonols, flavanones, flavanols, isoflavones, chalconoids, and anthocyanidins. Studies have shown that some can inhibit hepatic enzyme actions, show favourable effects in carbohydrate and lipid metabolism, delay diabetes onset and complications (Bahadoran *et al.*, 2013; Abbas *et al.*, 2016; Testa *et al.*, 2016; Al-Dosari *et al.*, 2017; Addepalli and Suryavanshi, 2018; Al-Ishaq *et al.*, 2019; Durazzo *et al.*, 2019).

Tannins are found in bark, leaves, fruits and seeds of plants. They are, however, commonly found in fruits, vegetables, and teas, contributing to some of the distinct flavours and medicinal potentials of some fruits, seeds, nuts and vegetables (Das *et al.*, 2020). Due to their variation in chemical structure, they are divided into four groups, namely condensed tannins, complex tannins, phlorotannins, and hydrolysable tannins (Al-Salih, 2010). The diabetes-related medicinal properties of tannins have been documented (Ajebli & Eddouks, 2019). Tannins from plants, including fruits and vegetables, have been shown to inhibit carbohydrate-digesting enzymes, suggesting their potential to control postprandial blood glucose levels (Al-Salih, 2010; Kunyanga *et al.*, 2011; Akhtar *et al.*, 2015; Cardullo *et al.*, 2020). In fact, some dietary hydrolysable tannins, including galotannins and ellagitannins have been shown to inhibit α-amylase and regulate the levels of blood glucose, while exhibiting potent antioxidant action (McDougall & Stewart, 2005; Xiao *et al.*, 2013; Cardullo *et al.*, 2020; Ćorković *et al.*, 2022; Mashile *et al.*, 2024; Setlhodi *et al.*, 2024).

Meanwhile, pomegranate is one of the few fruits with notable contents of hydrolysable tannins. A wealth of evidence has supported the cardiovascular and antioxidative health benefits of pomegranate fruit consumption, which has been majorly linked to the ellagitannins (punicalagins, ellagic acid and related phytochemicals), and some proanthocyanidins present in the fruit (Laurindo *et al.*, 2024). This study therefore focuses on this fruit.

2.7 Pomegranate

Pomegranate (*Punica granatum* L.) is a shrub or small tree that it belongs to Lythraceae family (**Table 2.1**). The tree can grow up to 10 meters (**Figure 2. 9a**), bearing an oval reddish-purple coloured fruit (**Figure 2. 9b**) (Holland *et al.*, 2009, Valero-Mendoza *et al.*, 2023; Ezeora *et al.*, 2024). Pomegranate is native to Iran, and it is widely cultivated in subtropical and tropical regions in countries in Asia, Africa, Australia, Europe, South and North America. The plant is known to adapt to different types of climatic conditions, and thus it is widely cultivated (Teixeira da Silva *et al.*, 2013). India has been at the top of the list of countries that cultivate pomegranate (Teixeira da Silva *et al.*, 2013; Mohammadi *et al.*, 2023; Ezeora *et al.*, 2024). In the Southern Hemisphere, South Africa and Peru are the leading producers (Brodie, 2009; Venkitasamy *et al.*, 2019; Ezeora *et al.*, 2023). There are more than 500 varieties of pomegranate. The most popular varieties in South Africa are the "Acco", "Wonderful", and "Herskovitz". "Wonderful" cultivar is the mostly grown (Stover & Mercure, 2007; Jalal *et al.*, 2018; Kahramanoğlu, 2019).

Table 2.1: Taxonomic classification of Pomegranate.

Classification	Name
Kingdom	Plantae – Plants
Sub-kingdom	Tracheobiontan – Vascular plants
Sub-division	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plant
Class	Magnoliopsida – Dicotyledons
Sub-class	Rosidae
Order	Myrtales
Family	Punicaceae(formerly), Lythraceae (currently)
Genus	Punica L.
Species	Punica granatum L. Punica protopunica Balf

USDA, 2014

Figure 2.9: Image showing (**a**) pomegranate tree with fruits and (**b**) pomegranate fruit

Pomegranate fruit comprises of 50% edible parts and 50% peel (Fawole and Opara, 2013). The edible parts are divided into the juice, which is 78% w/w and seed 22% w/w. The arils are succulent seed/kernels, where the juice is obtained from (**Figure 2.10**) (Jalal *et al.*, 2018; Valero-Mendoza *et al.*, 2023; Ezeora *et al.*, 2024).

Figure 2.10: Pomegranate fruit and its different parts

Traditionally, the pomegranate has been used to treat several health problems, such as stomach aches and bacterial infections (Holland & Bar-Ya'akov, 2014). Its cardiovascular health benefits have also been documented, which have been linked to the ellagitannins (punicalagins, ellagic acid, and related phytochemicals), some proanthocyanidins, and phenolic acids present in the fruit (Laurindo *et al.*, 2024). Pharmacological studies have also shown the anti-inflammatory, antioxidant, antimicrobial, anti-fungal, anti-carcinogenic, cardio-protective, and

anti-diabetic potentials of pomegranate (Melgarejo-Sánchez *et al.*, 2021; Yan-hui *et al.*, 2022; Mohammadi *et al.*, 2023; Valero-Mendoza *et al.*, 2023).

2.7.1 Pharmacological potential of the pomegranate plant in metabolic disorders

Pomegranate contains a variety of phytochemicals (**Table 2.2)** that are believed to positively influence metabolic processes, and possess health-promoting potentials (Glazer *et al.* 2012; Melgarejo-Sánchez *et al*., 2021).

Table 2.2: Major phytochemicals in different parts of pomegranate

Pomegranate parts	**Phytochemicals**
Flowers	Gallic and ursolic acid, as well as triterpenoids as asiatic acid and maslinic acid
Leaves	Tannins, flavonoids, piperidine alkaloids, ellagic acids, and esters of ellagic acids with glucose
Bark and roots	Alkaloids (especially piperidine alkaloids), ellagitannins (punicalin and punicalagin)
Seed	Punicic acid, oleanolic, palmitic, linolenic acid
Juice	Tannins, ellagitannins (punicalin and punicalagin), flavonoids (anthocyanins, flavonols and flavan-3-ols)
Peel	Phenolic acids, ellagitannins, flavonoids, and several minerals

Source: Magangana, *et al.,* (2020)

Pharmacological studies have been done on the flower, leaves, arils, and peel of pomegranate. In one of the studies, the methanolic extract of the pomegranate flower caused antihyperglycemic effects in Zucker diabetic fatty rats by modulating insulin sensitivity and the expression of glucose transporter type 4 (Huang *et al.*, 2005). In another study, the ethanolic extract of pomegranate flower demonstrated antioxidant, anti-dyslipidaemic, and antihyperglycaemic effects in high-fat-diet-induced and STZ-induced diabetic rats (Manoharan *et al.*, 2009; Tang *et al.*, 2018). The leaf extracts of pomegranate have also shown bioactive effects. A polyphenol-rich fraction and a hydroalcoholic extract of the leaves inhibited the the

activity of pancreatic lipase and reduced the intestinal absorption of fats (Adnyana, et al, 2014; Yu *et al.*, 2017), suggesting anti-obesogenic potential.

Studies have shown that pomegranate fruit juice has the potential to ameliorate oxidative and metabolic diseases. Several studies have documented the ameliorative potential of the fruit juice and extracts (ethanol, methanol, and water) on diabetic oxidative stress. The fruit juice and extracts increase glycogen content and insulin secretion, reduce blood glucose, improve glucose tolerance, lipid profile, modulate insulin signalling factor expression, reduce lipid peroxidation, and hepatic damage in animal models showing diabetic oxidative stress (Das *et al.*, 2001; Chidambara *et al.*, 2002; Das and Sama, 2009; Prasetyastuti *et al.*, 2014; Salwe *et al.*, 2015; Nguyen Thanh *et al.*, 2019; Gharib & Kouhsari, 2019).

Furthermore, a study in high-fat-diet fed rats showed that the juice of pomegranate fruit reduced weight gain, lowered blood glucose levels, improved insulin secretion and satiety, and modulated the mRNA expression of several enzymes metabolising lipid and glucose (Ahmed *et al.*, 2015). In vitro study, an extract or fraction of methanolic and hydro-ethanol nature of the whole fruit demonstrated lipase (Shamsiya *et al.*, 2016), and α-amylase inhibitory (Jain *et al.*, 2012; Redha *et al.*, 2018) activity, suggesting the anti-obesogenic and postprandial glycaemic control potential of the fruit. A recent study has shown that pomegranate peel can inhibit lipase activity, lipid peroxidation, glucose-induced protein, and modulate glucose uptake (Chukwuma *et al.*, 2020).

Advances in research have likewise revealed that the peel of pomegranate is rich in these bioactive ellagitannins and some proanthocyanidins, even more than in the edible part of the fruit (Siddiqui *et al.*, 2024; Setlhodi *et al.*, 2024; Mashile *et al.*, 2024), making the peel of pomegranate a promising source of ellagitannins.

2.7.2 Bioactive potential of pomegranate peel in oxidative and metabolic diseases

Pomegranate peel has been shown, in vitro, to be a potent scavenger of ROS and radicals, including being a potent iron chelating agent, as indicated by its ability to subdue ROS's deleterious nature (Middha *et al.*, 2013; Hernández-Corroto *et al.*, 2018; El-Hadary & Ramadan, 2019; Chukwuma *et al.*, 2020). **Table 2.3** shows the pomegranate peel's antioxidant and metabolic bioactivities.

Table 2.3: The bioactive potential of pomegranate peel in metabolic and oxidative diseases

Material, extract or isolate used	**Pharmacological action**	**References**
Peel powder	25 days of feeding a diet containing 1 and 5% peel powder protected against dextran sulphate sodium-induced intestinal and hepatic oxidative stress in rats by reducing small intestinal and hepatic LPO and increasing GSH content and GSH/GSSG ratio	Al-Gubory *et al.*, 2021
Ethanol extract	4 wks adm. (250 mg/kg bw, p.o.) of extract in female SHR reduced SBP and coronary artery ACE activity and improved the histology of the coronary artery.	Dos Santos *et al.*, 2016
Peel extract (255 mg is equivalent to 90 mg of ellagic acid)	8 wks adm. (255 mg, p.o.) of extract in patients undergoing hemodialysis improved renal inflammatory status and endothelial functions by reducing serum CRP, IL-6, ICAM-1, VCAM-1 and TNF-α.	Jafari *et al.*, 2020
Polysaccharide isolate	28 days adm. (100 – 400 mg/kg bw, p.o.) of isolation in cyclophosphamide-induced immunosuppressed mice increased splenic LDH and acid phosphatase activity, lymphocytes proliferation, immunoglobins and cytokines secretion, and antioxidant status.	(Wu *et al.*, 2019)
Ethanol extract	2 wks adm. (100mg/kg bw, p.o.) of extract protected against vancomycin-induced liver and kidney injury in male SD rats by reducing serum biomarkers of kidney and liver damage, tissue LPO, ROS production, inflammatory response, and pro-apoptotic processes, and improving	El Bohi *et al.*, 2021

Material, extract or isolate used	Pharmacological action	References
	renal and hepatic tissue histology and antioxidant status.	
Compound (Valoneic acid dilactone) isolated form the ethyl acetate fraction of the peel methanol extract and the extract	• In vitro inhibition of α-amylase, aldose reductase, and PTP1B. 21 days oral adm. of extract (200 na 400 mg/kg bw) and isolated compound (10 – 50 mg/kg bw) reduced FBG and improved GT and pancreatic histology	Jain *et al.*, 2012
Peel powder	4 wks adm. (500 and 1000 mg/kg bw, p.o.) of peel powder in HFD-fed albino rats reduced serum cholesterol, ALT, AST, CRP, IL-6, albumin, and creatinine, and protected against aortic histopathological degeneration.	(Salama *et al.*, 2021)
Acetone extract	• In vitro DPPH and NO radical scavenging activity • In vitro Fe^{3+} reducing activity • In vitro inhibition of glucose-induced BSA glycation • In vitro α-amylase inhibitory activity • Pancreatic lase inhibitory activity • Increased GSH and reduced LPO in hepatocytes induced with oxidative stress Increased glucose uptake in hepatocytes	(Chukwuma *et al.*, 2020)

Material, extract or isolate used	Pharmacological action	References
Aqueous extract and protein isolates	• In vitro ABTS and hydroxyl radicals scavenging activity • In vitro ACE inhibitory activity In vitro cholesterol esterase inhibitory activity	(Hernández-Corroto *et al.* 2018)
Water and methanol extracts	• In vitro DPPH radical and H_2O_2 scavenging activity In vitro Fe^{3+} reducing activity	(Middha, *et al.*, 2013)
75% methanol extract	• In vitro DPPH and ABTS radicals scavenging activity 56 days adm. (200 mg/kg bw, p.o.) of extract reduced HbA1c and BG, improved lipid profile, and serum biochemical markers of kidney and liver damage in alloxan-induced diabetic and HFD-fed male Wistar rats	El-Hadary & Ramadan, 2019

Abbreviations: Adm., administration; ACE, angiotensin converting enzyme; BSA, bovine serum albumin; BG, blood glucose; CRP, C-reactive protein; FBG, fasting blood glucose; GSH, reduced glutathione; GSSG, oxidized glutathione; GT, glucose tolerance; HbA1c, glycated haemoglobin; HFD, high-fat-diet; LDH, lactate dehydrogenase, LPO, lipid peroxidation; PTP1B, Protein tyrosine phosphatase 1B; ROS, reactive oxygen species; SBP, systolic blood pressure; SD, Sprague Dawley; SHR, spontaneously hypertensive rats.

Pomegranate peel has shown various pharmacological potentials in metabolic and cardiovascular disorders. These include anti-hyperglycaemic, anti-obesogenic, antiglycation, anti-hypertensive, and anti-atherogenic potentials. In vitro studies have shown the potential of pomegranate peel to inhibit enzymes and biological processes linked to diabetes, hypertension, obesity, dyslipidaemia, and associated oxidative complications via exerting inhibitory action on protein-tyrosine phosphatase 1B, α-amylase, glucose-induced protein glycation, aldose reductase, pancreatic lipase, angiotensin converting enzyme, and cholesterol esterase (Jain *et al.*, 2012; Hernández-Corroto *et al.*, 2018; Chukwuma *et al.*, 2020). In Chang cells, extracts of

pomegranate peel inhibited lipid peroxidation and modulated glucose uptake, suggesting antioxidant and glycaemic control potential (Chukwuma *et al.*, 2020; Akuru *et al.*, 2022).

In vivo, the glycaemic control and tissue-protective potentials of pomegranate peel have been demonstrated. With potency comparable to that of glibenclamide, pomegranate peel exerted blood anti-hyperglycaemic effect in alloxan-induced and high-fat-diet diabetic rat models, suggesting its antidiabetic potential (El-Hadary & Ramadan, 2019). Also, pomegranate peel was shown to exhibit tissue protective tendency by suppressing dextran sulfate sodium-induced intestinal and hepatic oxidative stress and tissue damage in rats (Al-Gubory *et al.*, 2021). In a different study, the ethanol extract of the peel demonstrated cardioprotective and anti-hypertensive properties through reducing coronary artery ACE activity and systolic blood pressure in rat models (dos Santos *et al.*, 2016). The study reported by Salama *et al.* suggests pomegranate peel has cardioprotective effects in high-fat-diet rats, as peel powder (500 and 1000 mg/kg bw for 28 days) it reduces alanine transaminase (ALT), aspartate aminotransferase (AST), serum cholesterol, C-reactive proteins, creatine, albumin, and interleukin-6, and protects against aortic histopathological degeneration (Salama *et al.*, 2021). In Sprague Dawley rats, the ethanol extract of pomegranate peel ameliorated vancomycin-induced kidney and liver injury by reducing biomarkers of kidney and liver damage, oxidative stress, pro-apoptotic activity, and inflammatory responses, as well as improving the histological structure in both renal and hepatic tissues (El Bohi *et al.*, 2021).

Furthermore, the immunomodulatory potential of pomegranate peel has been documented. Polysaccharides isolated from pomegranate peel (100 – 400 mg/kg bw for 28 days) potentiated immunomodulatory effects in immunosuppressed mice by increasing acid phosphatase and splenic lactate dehydrogenase activity, cytokines and immunoglobulins secretion, lymphocytes proliferation, and antioxidant status (Wu, *et al.*, 2019).

In clinical settings, pomegranate peel has also shown prospects of metabolic and vascular health benefits. Patients undergoing haemodialysis were given 255 mg of pomegranate peel extract daily for 8 weeks. The treatment outcomes suggest improvement in renal inflammatory status and endothelial function, due to reductions in serum IL-6, C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and TNF-α (Jafari *et al.*, 2020). In another clinical study, it was observed that a hydroalcoholic extract of pomegranate peel had favourable effects on the metabolic and vascular indices of T2D patients (Grabež *et al.*, 2020). This is because oral administration (twice daily for 8 weeks)

of a 250 mg capsule of the peel extract notably decreased blood pressure (systolic and diastolic), as well as detrimental blood lipids (triglycerides and LDL-cholesterol), and HbA1c levels, while notably increasing HDL-cholesterol levels.

The pomegranate peel has most phenolic components, which include phenolic acids, flavonoids, and tannins (Setlhodi *et al.*, 2024; Mashile *et al.*, 2024). Flavonoids, such as proanthocyanidins and hydrolysable tannins, including ellagitannins and gallotannins are the predominant or notable polyphenols subclasses in pomegranate peel, which have been linked to the afore-mentioned medicinal properties of pomegranate (Setlhodi *et al.*, 2024; Mashile *et al.*, 2024). The punicalagins are the main ellagitannins in pomegranate peel, which can undergo hydrolysis to release ellagic acid (Zuccari *et al.*, 2020) (**Figure 2.11**).

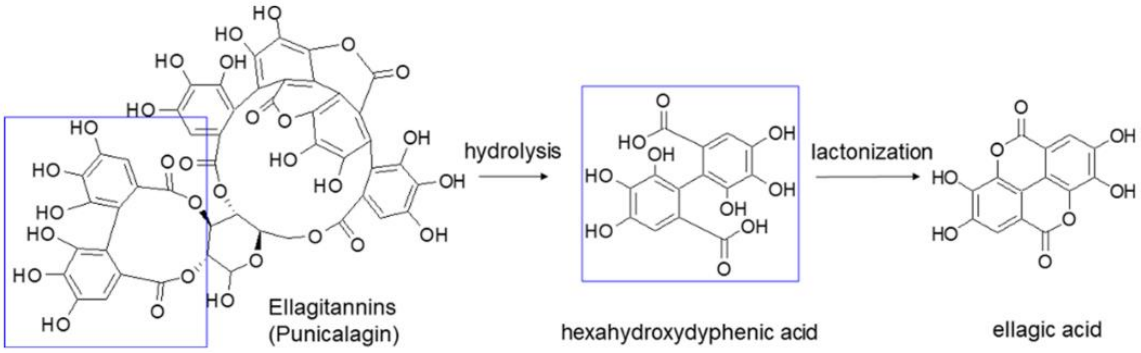


Figure 2.11: Chemical structure and hydrolysis of punicalagins (Zuccari *et al.*, 2020)

2.7.3 Pomegranate ellagitannins and their medicinal potential in metabolic diseases

As mentioned above, pomegranate peel contains tannins, and the most abundant tannins are the hydrolysable tannins, such as ellagitannins (Setlhodi *et al.*, 2024; Mashile *et al.*, 2024). Ellagic acid and punicalagin are the main ellagitannins found in pomegranate peel and have been shown to exhibit glycaemic control and antioxidant effects. These compounds are considered as key phytoconstituents responsible for many of the metabolic and oxidative health benefits attributed to pomegranate. When incorporated as a dietary supplement in overweight individuals, ellagitannins have demonstrated inhibitory effects on lipid peroxidation (Heber *et al.*, 2007). Type 2 diabetic (T2D) patients who received 180 mg of ellagic acid for 8 weeks exhibited significant reductions in blood glucose, detrimental blood lipids (triglycerides, total cholesterol and LDL-cholesterols), lipid peroxidation, pro-inflammatory markers (CRP, TNF-α, and IL-6) and insulin resistance, as well as appreciable increases in the activity of antioxidant

enzymes (Ghadimi *et al.*, 2021), suggesting antidiabetic and antioxidant potential of ellagic acid.

In studies conducted both vitro and in vivo, the antioxidant potential of commercial punicalagin has been demonstrated, which outperformed commercial punicallin and ellagic acids (Sun *et al.*, 2017; Xu *et al.*, 2021; Setlhodi *et al.*, 2024). Ellagitannins-rich fractions from pomegranate peel exhibited potent in vitro radical scavenging and anti-lipid peroxidative effects (Setlhodi *et al.*, 2024). Commercial ellagic acid has been shown to exhibit antioxidative, anti-lipogenic, and anti-dyslipidaemic activity in the liver of mice (Xu *et al.*, 2021). This is because 14 days oral administration of ellagic acid (0.1% and 0.3%) to C57BL/6J mice improved hepatic lipids profile (reduced total and LDL cholesterol levels and increased HDL-cholesterol level), suppressed hepatic lipogenic and pro-inflammatory proteins/markers, reduced hepatic lipid peroxidation, and increased antioxidant enzymes activity (Xu *et al.*, 2021). Additionally, HepG2 cells treated with ellagic acid showed improvement in high glucose-induced insulin resistance and oxidative stress (Ding *et al.*, 2019). Punicalagin and ellagitannins-rich fraction from pomegranate peel have been reported to be an effective inhibitor of α-amylase (Mashile *et al.*, 2024), suggesting its glycaemic control potential.

2.8 Problem statement

Metabolic diseases, including T2D diabetes and the associated vascular complications, continue to plaque the global population. Recent statistics have shown the increasing prevalence of metabolic and cardiovascular diseases and the associated mortality outcomes, posing global socioeconomic burdens. Unfortunately, the increasing prevalence of obesity and some unhealthy lifestyle factors, such as physical inactivity and unhealthy dieting, has exacerbated the prevalence of metabolic and cardiovascular diseases. This scenario increases the risk of metabolic syndrome, a set of metabolic disturbances that are associated with cellular insulin resistance and dyslipidaemia, which increases the risk of T2D and cardiovascular diseases. In fact, documented evidence has shown that consumption of diets high in saturated fats, trans fats, and refined carbohydrates, including added sugars (sucrose and fructose) increases the risk of metabolic syndrome. For example, chronic consumption of fructose has been shown to cause hepatic insulin resistance, proinflammatory responses, increased lipogenesis and visceral adiposity, dyslipidaemia, and increased oxidative stress, which are pathological alterations that promote metabolic syndrome. The metabolic and cardiovascular

health benefits of pomegranate fruit have been documented, which have been mainly attributed to the flavonoids, such as proanthocyanidins and hydrolysable tannins, including ellagitannins and gallotannins, contained in the fruit. Recent research advances have shown that the peel of pomegranate is also rich in ellagitannins, and studies suggest that the peel and its ellagitannin constituents may possess ameliorative potential against metabolic and cardiovascular impairments, including diet-induced metabolic and cardiovascular disorders. However, it remains unclear whether ellagitannins from pomegranate peel can mitigate or suppress the detrimental effects caused by high fructose consumption.

2.9 Aim and objectives

The aim of this study was to investigate the effect of an ellagitannins-rich fraction obtained from pomegranate peel on metabolic alterations related to T2D in a fructose-fed experimental animal model.

The objectives include the following:

i. Recovery and LC-MS characterization of ellagitannins-rich fraction from pomegranate peel.
ii. Determine its *in vivo* effect on fructose-induced metabolic alterations related to T2D.

CHAPTER 3

MATERIALS AND METHODS

3.1 Procurement of pomegranate fruit and preparation of crude peel extract

Class 1 or processing grade of pomegranate fruit (Var. "Wonderful") was purchased from Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa) in April 2024. The fruits were washed with distilled water, and the fruit peel was obtained. The peel was incubated in distilled water for 1 h at a ratio of 1:3 mass (kg)/volume (L). The incubation was done in an FSOH thermostat oven (Labcon, Krugersdorp, South Africa) set at 65 °C. After the 1 h incubation, the peel was macerated in the water used for the incubation. The macerated peel was first filtered using a cheese cloth, and thereafter, the filtrate was filtered twice using a 500 mm diameter Grade 1 Whatman® qualitative filter paper (Merck, Johannesburg, South Africa). The filtered extract was freeze dried using a Martin Christ Alpha 1-2 LDplus Freeze Dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The vacuum pressure for freeze drying was lower than 1 mbar, while the temperature of the condenser was between 52 and 56 °C. The extract was stored in a lidded glass vial at -20 °C.

3.2 Preparation of ellagitannins-rich fraction from the peel extract

To prepare the ellagitannins-rich fraction, a previous method (Seeram *et al.*, 2005) was modified and used (**Figure 3.1**). An Amberlite® XAD16N resin (product code: XAD16; Merck, Johannesburg, South Africa) was used to recover the ellagitannins-rich fraction from the peel. The resin was first prepared and equilibrated before use. To do this, approximately 45 g of the resin was washed three times with methanol and rinsed four times with distilled water. The resin was loaded into a column connected to an MD 1C VARIO vacuum pump (VACUUBRAND GMBH + CO KG, Wertheim, Germany). In the column, the rinsed with distilled water to remove any residual methanol and then equilibrated in distilled water for 12 – 16 h. Next, 5 g of the freeze-dried peel extract was dissolved in 250 mL of distilled water and filtered with a Whatman filter paper to remove any particles. After the 12 – 16 h equilibration, the water was removed from the column by applying about 100 mbar vacuum pressure. Next the dissolved extract was gently poured onto the resin in the column so that the ellagitannins can be adsorbed into the resin. The column was eluted with distilled water using the same vacuum pressure. Elution continued until the eluent was consistently colourless, indicating that

all the tannins have been absorbed into the resin (**Figure 3.1**). The vacuum pressure was used to remove residual water in the column. Next, the adsorbed ellagitannins/tannins was gradually eluted with methanol using a vacuum pressure of 200 mbar. The eluent, which is the ellagitannins-rich fraction, was concentrated using a Buchi Rotavapor® R-300 (BÜCHI Labortechnik AG, Flawil, Switzerland), diluted 10x with distilled water and freeze-dried to remove any methanol residue. The Freeze-dried ellagitannins-rich fraction was stored in lidded vials at -20 °C.

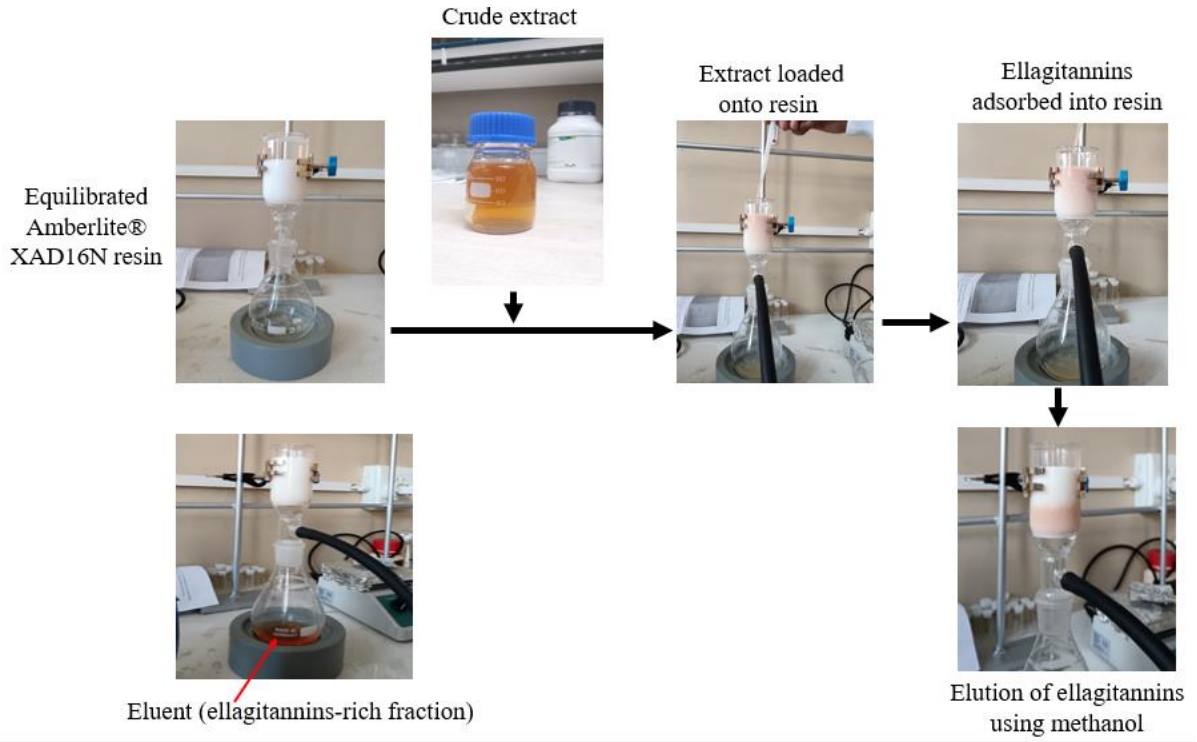


Figure 3.1: Schematic diagram shown the recovery of ellagitannins-rich fraction

3.3 LC-MS analysis of the ellagitannins-rich fraction

The liquid chromatography mass spectrometry (LC-MS) analysis was commercially outsourced from the Central Analytical Facility at Stellenbosch University, Stellenbosch, Western Cape, South Africa. The LC-MS was performed on A Waters Synapt G2 (Waters Corporation, MA, USA) that is equipped with ESI probe and ESI Pos. at a capillary and cone voltage of 2500 and 15 V, respectively. The scan ranged from 100 to 15000 Da. An Acquity binary solvent manager was used to operate the liquid chromatography. The chromatographic separation was done on a HSS T3 column at 0.25 mL/min flow rate and 30 °C temperature using a gradient mobile phase condition. The column had 2.1 x 150 mm dimension and 1.8 µm

particle size (Waters Corporation, MA, USA). The mobile phase system comprised two solvents, namely solvents A (water containing 0.1% methanoic acid) and B (acetonitrile containing 0.1% methanoic acid). The sequence of the gradient mobile phase condition was 100% of A and 0% of B for 10 min, 72% of A and 28% of B for 21 min, 60% of A and 40% of B for 50 sec and 0% of A and 100 % of B for 2 min. The sample were prepared by dissolving in a mixture of acetonitrile and water (1:1 v/v) and filtering using a 0.22 µm filter. The injection volume of the sample was 20 µL. A 254 nm wavelength was used to monitor and record the signals of the separated compounds. The compounds in the fraction were tentatively identified using accurate masses and elemental compositions according to the extracted ion. The compounds were semi-quantified in mg/Kg relative to a catechin calibration curve. The LC-MS analysis of the fraction was done in duplicate, and the amounts (mg/Kg) of the identified compounds were presented in the mean ± SD of duplicate analysis.

3.4 Experimental animals

Animal research ethics approval was obtained from the University of the Free State Animal Research Ethics Committee (reference number: UFS-AED2024/0053; date: 01 October 2024). Eighteen 14 weeks old Sprague-Dawley rats were obtained from the University of the Free State Animal Research Centre. The animals were kept in large-sized cages to acclimatize for 5 days. The cages had the following dimensions: 598 mm (L) x 380 mm (W) x 200 mm (H) with Grid top hatch access of 220 mm (L) x 225 mm (W) x 70 mm (H). Each cage contained two animals. The condition of the room where the animals were housed was controlled as follow: 20 – 24 °C (set point at 22 °C) temperature, 40-70% (set point at 60%) humidity and a 12 h light-dark cycle. All the protocols of the animal experiment were done in accordance with the rules and regulations of the of the University of the Free State Animal Research Ethics Committee, which aligns with the guidelines stipulated by SANS 10386:2008 (the South African National Standard for the Care and Use of Animals for Scientific Purposes).

3.5 Animal grouping and treatments

The animals were randomly divided into three groups, namely “Normal Control”, “Fructose” and “Fructose + Ellagitannins” groups. Each group had six animals. There were two animals in each cage. The animals in the “Normal Control” group received normal drinking water *ad*

libitum for six weeks experimental period. The animals in "Fructose" group received 20% fructose solution instead of drinking water for the six weeks experimental period, while the animals in the "Fructose + Ellagitannins" group received 20% fructose solution containing the ellagitannins-rich fraction instead of drinking water for the six weeks experimental period. A 20% fructose solution has been previously used to induce metabolic alterations that are consistent with this study (Severcan *et al*., 2021; Liu *et al*., 2022). Hence, an equivalent fructose concentration was adopted in the present study. The study ensured that the animals in the "Fructose" and "Fructose + Ellagitannins" groups consumed approximately equal volumes of fructose solution. To achieve this, each cage in "Fructose" group was supplied daily with 110 mL of 20% fructose solution daily. Equal volume of 20% fructose solution containing the ellagitannins-rich fraction was also supplied to each cage in "Fructose + Ellagitannins" group. The amount of the ellagitannins-rich fraction in the fructose solution was predetermined and calculated such that each animal will receive approximately 200 mg/kg bw upon complete consumption of fructose solution. This was reviewed weekly, based on the animals' body weight. It is important to note that throughout the experimental period, the rats in the "Fructose" and "Fructose + Ellagitannins" groups consumed at least 96% of the 110 mL volume supplied to each cage. A 200 mg/kg bw dose was chosen, because a study on HFD/STZ-induced mice (Jin *et al*., 2020) showed that a similar dose of punicalagin (the most abundant ellagitannin of the tested fraction) had positive effects on biochemical parameters consistent with our study

3.6 Weekly measurement of food intake and body weight

At the start of the experiment, all the animals were weighed before being assigned to the different groups. Thereafter, their weight was measured weekly on the last day of each week. The weight gain was determined by subtracting the initial weight of the animals from the final weight at the end of the six-week experimental period. Food intake was measured on the last day of each week, throughout the six-week experimental period.

3.7 Measurement of blood glucose and oral glucose tolerance

Oral glucose tolerance was measured on the 3rd day of the 6th week of the experimental period. On the day of the oral glucose tolerance test, food was removed at 3:00 AM. After 7 hours (i.e., at 10:00 AM), the tail-tip blood glucose level (mmol/L) of the animals was measured using a

Contour Plus Blood Glucose Monitoring System (Ascensia Diabetes Care Holdings AG, Basel, Switzerland). This measurement represented the blood glucose level at 0 min. Immediately after the blood glucose measurement, the animals were orally administered with 2 g/kg bw of glucose. Post glucose administration, the blood glucose level of the animals was measured at every 30 min interval until 120 min. The area under the curve was calculated using the following previously reported formular (Sakaguchi *et al*., 2015):

$$\text{AUC (mg.h/dL)} = \frac{\text{BG0} + (\text{BG30 x 2}) + (\text{BG60 x 3}) + (\text{BG120 x 2})}{4}$$

Where BG0, BG30, BG60 and BG120 mean blood glucose (mg/dL) at 0, 30, 60 and 120 min, respectively. To convert the blood glucose from mmol/L to mg/dL, a multiplication factor of 18.018 was used.

The tail-tip non-fasting blood glucose level of the animals was measured on the 5th day of the 6th week of the experimental period using a glucometer. The tail-tip fasting blood glucose of the animal was measured on the day the animals were euthanized, which was the following day after the six weeks experimental period. Food was removed at 3:00 AM. After 7 h (at 10:00 AM), the tail-tip blood glucose level (mmol/L) of the animals was measured using a glucometer. The blood glucose was.

3.8 Measurement of blood lipids

The blood lipids levels (mmol/L) were measured on the day the animals were euthanized using a Mission Lipogram Analyzer (ACON Laboratories, Inc, San Diego, California). First, the fasted animals were anaesthetized. Next, blood was collected via the lateral saphenous vein and used to measure the blood lipids. The blood lipids measured were triglycerides, total cholesterol, and HDL-cholesterol. LDL-cholesterol level was computed using Friedewald's formula (Friedewald *et al.*, 1972):

$$LDL_C\ (mmol/L) = T_C - HDL_C - \frac{TG}{2.2}$$

Where LDL_C is LDL-cholesterol level (mmol/L), T_C is total cholesterol level (mmol/L), HDL_C is HDL-cholesterol level (mmol/L), and TG is triglyceride level (mmol/L).

3.9 Animal euthanization, biological samples collection and preparation

The animals were euthanized by exposure to isoflurane gas. Thereafter, blood was collected by cardiac puncture and kept in tubes without an anti-coagulant. The liver tissue was also collected from the animals and frozen in liquid nitrogen, before storage at -80 °C. A small portion of the liver tissue was fresh liver tissue was immersed in a 10% neutral buffered formalin solution to preserve the tissue for histological examination.

The blood was immediately processed to recover the serum as follows. The blood was centrifuged at 3500 x g for 10 min at 4 °C using a NUVE NF 800R centrifuge (Separations, Johannesburg, South Africa). The serum, which was the clear upper layer was collected and stored at -80 °C.

The liver homogenate was prepared from the frozen liver tissue samples as follows. First, the tissue was thawed. Approximately 500 g of the tissue was homogenised in 5 mL of ice-cold 50 mM sodium phosphate buffer, containing 0.5% v/v Triton X-100 and 1 mM EDTA (pH, 7.4 – 7.5) using a D1000 handheld homogeniser (Merck, Johannesburg, South Africa). The homogenate was centrifuged at 10,800 x g for 10 min at 4 °C and the supernatant was aliquoted into microtubes and stored at -80 °C.

3.10 Measurement of protein concentrations

Protein concentration (mg/mL) was measured in serum and tissue homogenate using the bicinchoninic acid method. To estimate the protein concentration, 25 µL of the samples or bovine serum albumin (BSA; Merck, Johannesburg, South Africa) standards (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL in final assay volume) was mixed with 200 µL of protein quantification reagent in a clear-bottom transparent 96-well plate. The protein quantification reagent a 4% $CuSO_4.5H_2O$ solution (Merck, Johannesburg, South Africa) that has been diluted 50x with bicinchoninic acid solution or reagent (Merck, Johannesburg, South Africa). The plate was incubated in the dark for 30 min at 37 °C. After cooling to room temperature, absorbance was measured at 562 nm against a water blank using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA). The protein concentration (mg/mL) of the samples was extrapolated from BSA standard curve ($y = 0.5194x + 0.1586$; $R^2 = 0.9956$).

3.11 Measurement of liver glycogen content

The liver glycogen content was measured using the method reported by Lo *et al.* (1970) as modified by Izu *et al.*, (2024). First, the frozen liver was thawed. Approximately 0.5 g of the liver tissue was transferred into clean capped vials. Next, 1.5 mL of a 30% KOH solution was added. The vial was boiled for 40 min so that the tissue is disintegrated to form a homogenous solution. The homogenous solution was allowed to cool. After cooling, 2 mL of 95% ethanol solution was added. The vials were allowed to stand for 30 min at 2 – 8 °C, during which the sugar contained in the solution precipitates. The vials were centrifuged at 1200 x g for 30 min at room temperature and the sugar residue was recovered after decanting the supernatant. The sugar residue was washed 3x with 95% ethanol solution before dissolving in 3 mL of distilled water. In empty vials, a 200 µL aliquot of the sugar solution or glycogen (Merck, Johannesburg, South Africa) standard solutions (0.003125 – 0.2 mg/200 µL) was mixed with 1 mL of a 5% phenol solution (Merck, Johannesburg, South Africa) and 5 mL of H_2SO_4 (98% solution). Next, a 250 µL from the mixture was transferred into designated wells of a clear-bottom transparent 96-well plate and absorbance was measured at 490 nm against a water blank. The glycogen content (mg/g tissue) was computed from a glycogen standard curve ($y = 4.4755x – 0.0102$; $R^2 = 0.9987$) using the following formula:

$$Tissue\ glycogen\ content\ (mg/g\ tissue) = \frac{C * D}{M}$$

Where, "C" is the extrapolated concentration (mg/200 µL); "D" is the dilution factor = 31, which is the dilution of 200 µL of the dissolved residue in 6.2 mL of assay volume; "M" = 0.5 g, which is the mass (g) of tissue used in the assay.

3.12 ELISA-based quantification of serum insulin, liver IL-1β and Akt phosphorylation

Serum insulin was quantified using a Rat/Mouse ELISA Kit (Cat. # EZRMI-13K, Merck, Johannesburg, South Africa). The serum insulin concentration (ng/mL) extrapolated from a curve ($y = 0.0768x – 0.004$; $R^2 = 0.9981$) of insulin standard concentrations (0.2, 0.5, 1, 2 and 5 ng/mL) versus the corresponding absorbances at 450 nm. The serum insulin concentration in ng/mL was converted to concentration of mU/L using a multiplication factor of 24.8 as documented in a Rat High Range Insulin ELISA protocol manual (ALPCO Diagnostics, Salem, New Hampshire, United States; https://s3.amazonaws.com/alpco-docs/80/80-INSRTH-E01,%20E10.pdf).

The liver interleukin 1β (IL-1β) concentration was measured using an Elabscience® High Sensitivity Rat IL-1β (interleukin 1 Beta) ELISA Kit (catalog No: E-HSEL-R0002, Biocom Africa, Centurion, Gauteng, South Africa). The liver IL-1β concentration (pg/mL) extrapolated from a curve ($y = 0.0448x - 0.0447$; $R^2 = 0.9987$) of IL-1β standard concentrations (0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 pg/mL) versus the corresponding absorbances at 450 nm.

Liver Akt phosphorylation was measured using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalog No: RAB0012, Merck, Johannesburg, South Africa). Using a colorimetric approach (absorbance at 450 nm), the kit was used to estimate the phospho-Akt and pan-Akt levels. The absorbance intensity corresponded to the levels of phospho-Akt and pan-Akt. The level of Akt phosphorylation was computed as the Phospho-Akt/pan-Akt ratio by using the ratio between the corresponding absorbance values.

3.13 Computation of homeostatic model assessment of insulin resistance

The homeostatic model assessment of insulin resistance (HOMA-IR) was computed using the fasting blood glucose (mmol/L) and fasting serum insulin (mU/L) levels of the animals according to the following previously reported formula (Vilela *et al.*, 2016):

$$HOMA - IR = \frac{Fasting\ blood\ glucose\ (mmol/L) * Fasting\ serum\ insulin\ (mU/L)}{22.5}$$

3.14 Measurement of lipid peroxidation

Lipid peroxidation was determined by measuring the level of thiobarbituric acid reactive substances (TBARS) as an equivalent of malondialdehyde (MDA) standards. The method reported by Izu *et al.* (2024) was used in performing this assay. Into designated vials, 100 µL the serum or tissue homogenate or MDA (Merck, Johannesburg, South Africa) standard solutions (7.5, 15, 22.5, 30, 45, and 60 µM) was added. Next, 500 µL of 0.25% thiobarbituric acid solution (Merck, Johannesburg, South Africa), 200 µL of 20% acetic acid solution (Merck, Johannesburg, South Africa) and 200 µL of distilled water were added into the vials. The vials were closed and placed in boiling water for 50 minutes. After cooling, a 250 µL aliquot form each vial was transferred into designated wells of a clear-bottom transparent 96-well plate and absorbance was measured at 532 nm against a water blank. The level of lipid peroxidation was

determined by extrapolating the equivalent MDA concentrations of the absorbances from an MDA standard curve (y = 0,0098x + 0,002; R2 = 0,9984).

3.15 Measurement of catalase and superoxide dismutase activity

The catalase activity was measured with an Elabscience® Catalase (CAT) Activity Assay Kit (catalog No: E-BC-K031-S, Biocom Africa, Centurion, Gauteng, South Africa), superoxide dismutase (SOD) activity was measured with a an Elabscience® Total Superoxide Dismutatse (T-SOD) Activity Assay Kit (WST-1 Method) (catalog No: E-BC-K020-M, Biocom Africa, Centurion, Gauteng, South Africa)

Catalase activity was determined by colorimetrically (at 450 nm) measuring the amount of residual H_2O_2 after monitoring the decomposition of H_2O_2 by catalase for 1 min.

$$Catalse\ activity\ (U/mg\ protein) = \frac{\Delta A * 32.5 * DF}{T * SV}$$

Where "ΔA" = Absorbance of control - Absorbance of test or sample; 32.5 is the reciprocal of H_2O_2 standard curve as mentioned in kit; "DF" is the dilution factor of sample; "T" is the 1 min time interval; "SV" is the sample volume in mL, which was 0.025 mL.

The superoxide dismutase activity of the serum and liver homogenate was measured by monitoring its inhibitory action on the colorimetric (at 450 nm) reduction of a superoxide ion-mediated reduction of a tetrazolium salt [2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. The inhibition was computed using the formula below.

$$Inhibition\ (\%) = \frac{OD_C - OD_S}{OD_C}\ x\ 100$$

Where "OD_C" is the blanked optical density for the control (without enzyme/sample), while "OD_S" is the blanked optical density for the samples.

The specific activity (U/mg protein) was calculated with respect to the protein concentration of the tissue homogenates as follows:

$$Specific\ activity\ (U/mg\ protein) = \frac{I * RV * DF}{50\% * SV * PC}$$

Where, “I” is the percentage inhibition (%) by enzyme on superoxide ion-mediated colorimetric reduction of tetrazolium salt; “DF” is the dilution factor of a sample before it is used in the assay; “RV” is the reaction volume of the assay in mL; “SV” is the sample volume in mL; “PC’ is the protein concentration (mg/mL) of the samples.

3.16 Examination of hepatic histology

The liver tissues were removed from the 10% neutral buffered formalin solution dehydrated by successive immersion in 50%, 70%, 90% and 100%, respectively. Next, the tissues were repeatedly immersed in xylene to remove any residual ethanol. Using a standard protocol, the tissues were embedded in paraffin, before cutting into thin sections of about 4 µm thickness. The sections were mounted onto a slide and stained with hematoxylin and eosin to expose key histological features. A DPX mounting media was applied to and a cover slip was mounted. Histological features were captured with a Leica slide scanner.

3.17 Data and statistical analyses

The data of this study was analysed using the 2016 version of MS Excel. Data were presented in “average ± SD” format of six animals per experimental group. Statistical analysis was carried out using the IBM SPSS software, Version 27 (IBM Corp, Armonk, NY, USA). For each parameter measured, the data means of the different experimental groups was compared using the one-way analysis of variance (ANOVA). The *post hoc* multiple comparison was done using the Tukey *post hoc*. Statistical significance between any two compared groups was set at $p < 0.05$. Different alphabetical letters between any two compared groups were used to denote statistical significance ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 Phytochemical profile of the peel's ellagitannins-rich fraction

LC-MS data showed the presence of mostly hydrolysable tannins, which include ellagitannins such as pendunculagin, punicalagin, corilagin, ellagic acid glucoside and eschweilenol C (**Figure 4.1** and **Table 4.1**). Punicalagin, corilagin and ellagic acid glucoside were more abundant than other ellagitannins. Catechin flavonoids, such as epigallocatechin and epicatechin where also present in the fraction. Epigallocatechin was more abundant than epicatechin. Other flavonoids detected include flavonoid-3-O-glycosides (astilbin and astragalin 7-rhamnoside) and flavonoid-7-O-glucuronides (3'-Methoxytricetin 7-glucuronide), which were not as abundant as epigallocatechin. With a concentration of 765 ± 68.8 mg/kg fraction, punicalagin was the most abundant compound in the ellagitannins-rich fraction, followed by ellagic acid glucoside (353 ± 29.7 mg/kg fraction). The punicalagin peak of (541.0267 Mz) at retention time of 6.24 min results from a fragmentation pattern as depicted in extracted ion chromatogram of punicalagin (**Figure 4.2**).

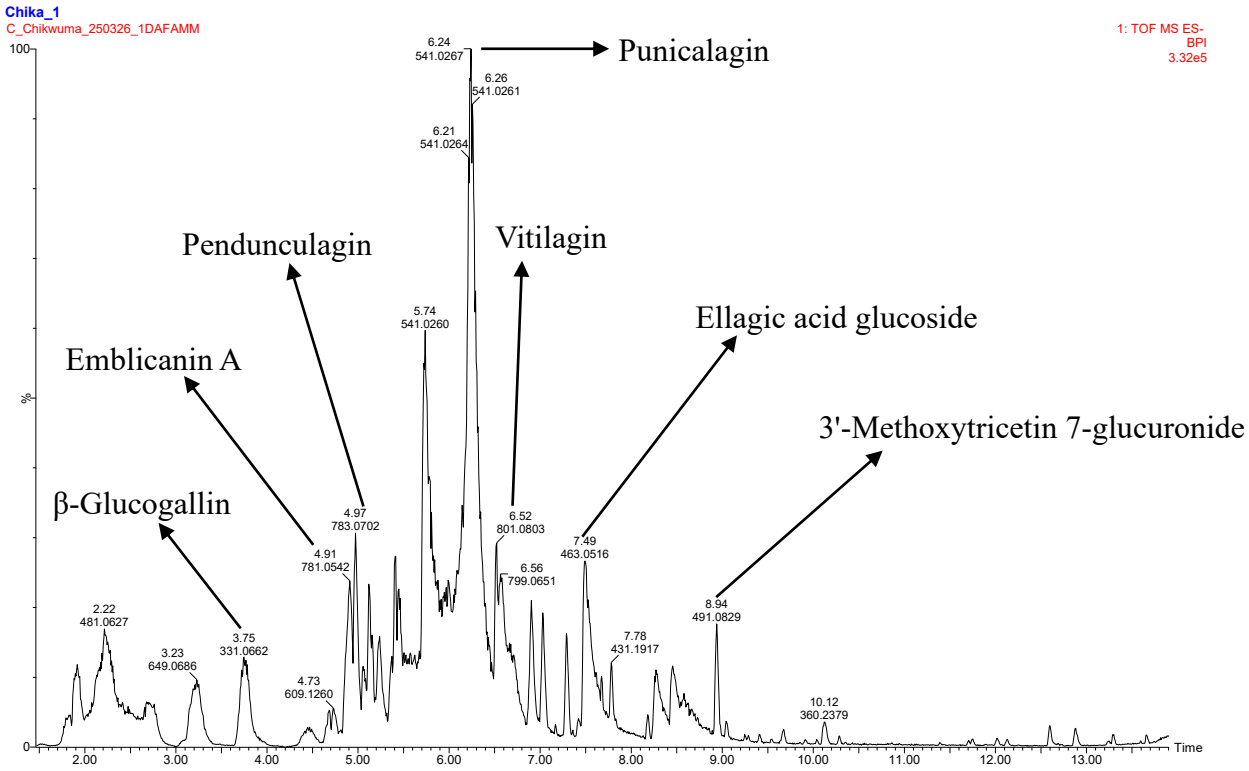


Figure 4.1: Annotated LC-MS chromatogram of the ellagitannins-rich fraction showing the peaks of the identified compounds.

Table 4.1: LC-MS data of the ellagitannins-rich fraction

Compound	Ontology	AMz	ART (min)	Total score	Amount (mg/kg) of compounds in fraction
β-Glucogallin	Tannins	331.0673	3.763	7.8439	170 ± 17.5
Emblicanin A	Hydrolyzable tannins	781.0549	4.916	5.8806	78.7 ± 6.04
Pendunculagin	Hydrolyzable tannins	783.0717	5.01	NA	105 ± 19.6
Epigallocatechin	Catechins	305.0667	5.413	7.2786	322 ± 24.3
Punicalagin	Hydrolyzable tannins	1083.062	5.735	5.592	765 ± 68.8
Vitilagin	Hydrolyzable tannins	801.0796	6.524	5.6299	54.8 ± 4.80
Digalloyl-hexahydroxydiphenoyl glucose	Hydrolyzable tannins	785.0859	6.911	NA	72.3 ± 6.21
Corilagin	Hydrolyzable tannins	633.0743	7.038	6.8868	223 ± 22.5
Epicatechin	Catechins	289.0714	7.287	6.6096	20.8 ± 1.89
Astilbin	Flavonoid-3-O-glycosides	449.1088	7.295	6.3386	183 ± 13.4
Ellagic acid glucoside	Hydrolyzable tannins	463.0522	7.502	5.4882	353 ± 29.7
Eschweilenol C	Hydrolyzable tannins	447.0564	8.463	6.2717	61.4 ± 14.5
3'-Methoxytricetin 7-glucuronide	Flavonoid-7-O-glucuronides	491.083	8.947	6.5177	54.2 ± 6.07
Astragalin 7-rhamnoside	Flavonoid-7-O-glycosides	593.1508	9.054	7.0527	41.2 ± 0.69

Abbreviation: ART, average retention time; AMz, average mass. The amount (mg/g fraction) of the identified compound is presented in "average ± SD" format of duplicate analysis.

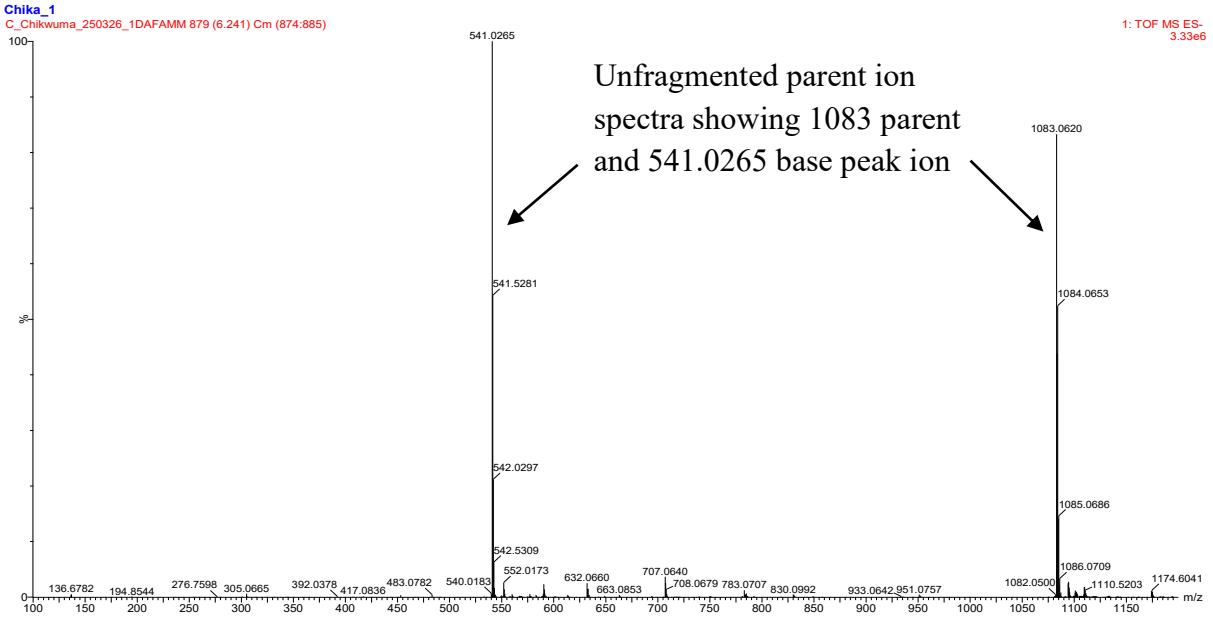


Figure 4.2: Extracted ion chromatogram of punicalagin showing the fragmentation pattern

4.2 Body weight gain and food intake of animals

Six weeks consumption of 10% fructose solution caused significantly ($p < 0.05$) more weight gain relative to the consumption of normal water (**Figure 4.3**). Conversely, six weeks of consuming a 10% fructose solution resulted in significantly ($p < 0.05$) lower food intake compared with the consumption of normal water (**Figure 4.3**).

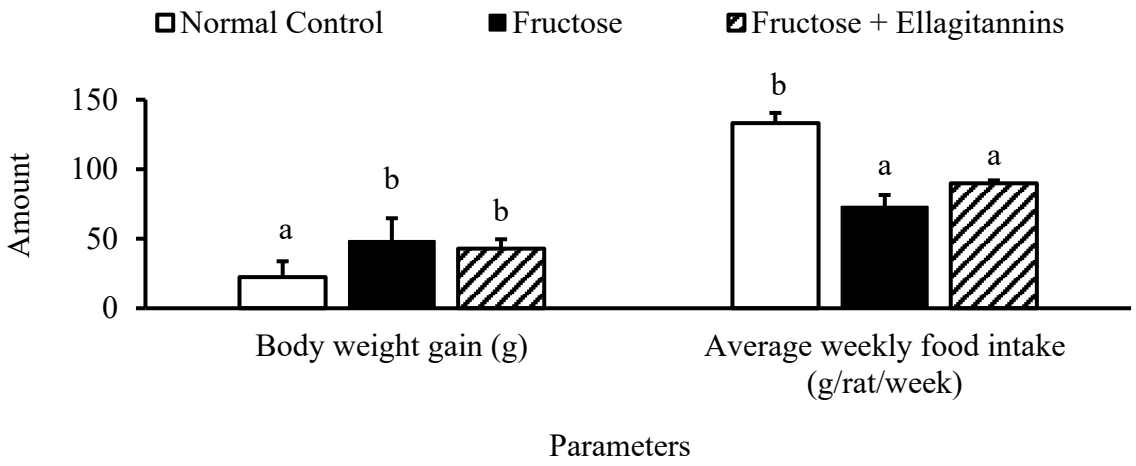


Figure 4.3: Body weight gain and food intake of animals after experimental period. Data are presented in "average ± SD" format of six animals. For each parameter, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

Treatment with the ellagitannins-rich fraction did not notably suppress the fructose-induced weight gain. However, although not significant ($p > 0.05$), treatment with the ellagitannins-rich fraction caused a notably increase in food intake relative to the absence of ellagitannins treatment intervention.

4.3 Blood glucose data of animals

The oral glucose tolerance capacity of the animals in the last week of the six weeks experimental period is presented in **Figures 4.4 and 4.5**. After 120 min of oral glucose administration, the average blood glucose level of the rats in the "fructose" group was significantly ($p < 0.05$) higher than the average blood glucose levels of the rats in the "normal control", and "fructose + ellagitannins" groups, although the rats in "normal control" group had a significantly ($p < 0.05$) lower average blood glucose level than the rats in the "fructose + ellagitannins" group.

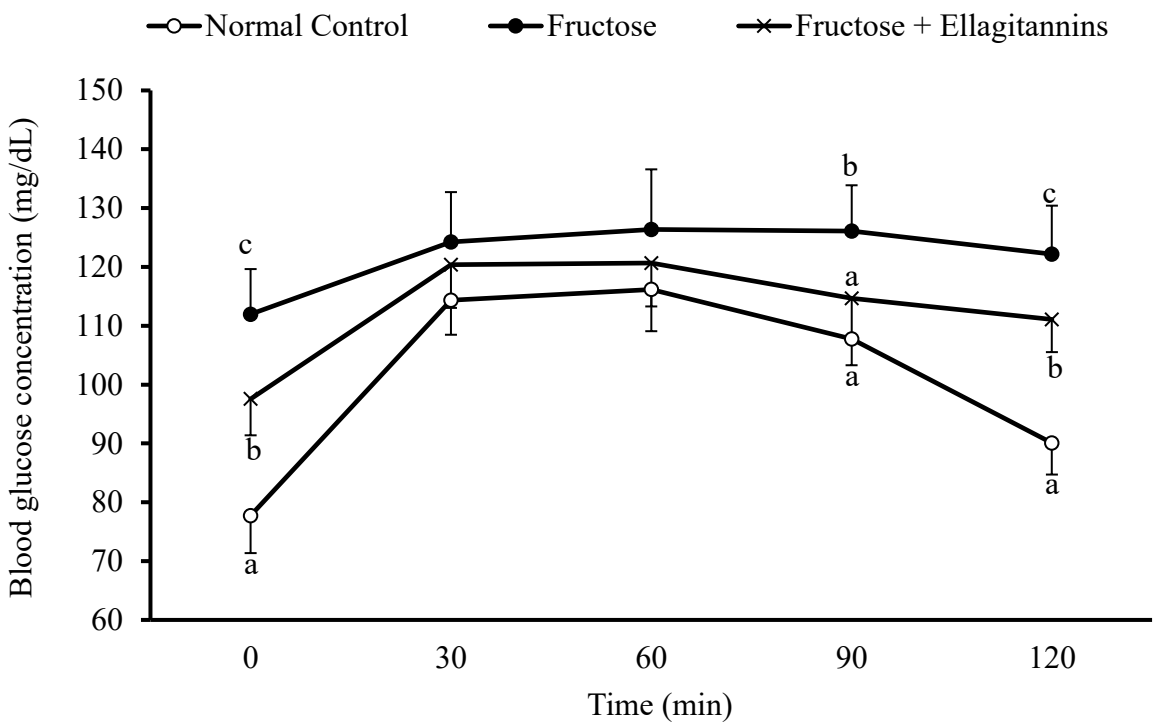


Figure 4.4: Blood glucose data at the different time points of the oral glucose tolerance test. Data are presented in "average ± SD" format of six animals. At a given time point, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

The area under curve (AUC) calculated from the OGTT plot or graph showed that the rats in the "normal control" group had a significantly ($p < 0.05$) lower average AUC (AUC = 209 ± 7.68 mg.h/dL) than the rats in the "fructose" group (AUC = 246 ± 15.5 mg.h/dL), suggesting that the prolonged consumption of 10% fructose significantly ($p < 0.05$) impaired postprandial glycaemic control (**Figure 4.5**). Although not significant ($p > 0.05$), the AUC data showed that treatment with the ellagitannins-rich fraction improved the impaired glucose tolerance caused by fructose consumption.

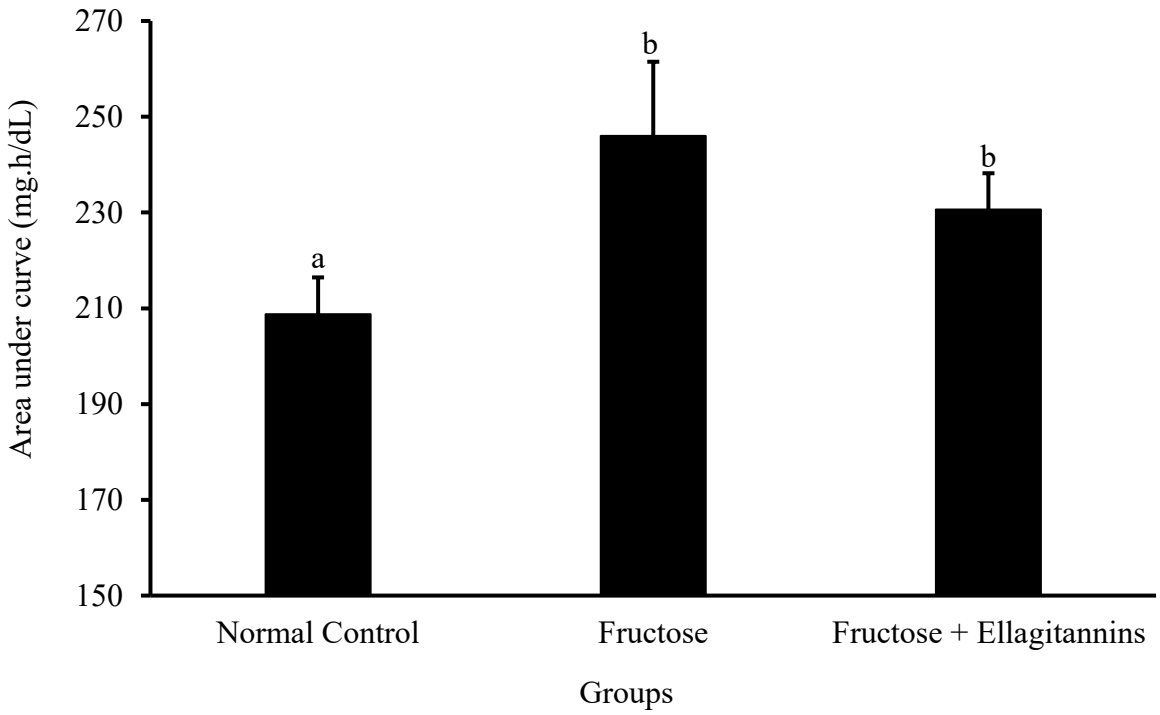


Figure 4.5: Computed area under curve of oral glucose tolerance plot/graph. Data are presented in "average ± SD" format of six animals. The data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

The non-fasting and fasting blood glucose levels of animals measured in the last week of the experimental period are presented in **Table 4.2**. The fructose-fed rats showed significantly higher non-fasting and fasting blood glucose levels compared with the "normal control" group ($p < 0.05$) suggesting impaired glycaemic control. Treatment with the ellagitannins-rich fraction normalise blood glucose levels but reduced non-fasting ($p > 0.05$) and fasting ($p < 0.05$) glucose levels in fructose-fed rats (**Table 4.2**).

Table 4.2: Blood glucose, serum insulin and liver glycogen levels of animals

Groups	Non fasting blood glucose (mg/dL)	Fasting blood glucose (mg/dL)	Serum insulin (mU/mL)	Liver glycogen content (mg/g tissue)
Normal Control	91.9 ± 8.05[a]	80.7 ± 7.24[a]	10.1 ± 0.95[a]	45.3 ± 3.65[a]
Fructose	116 ± 5.54[b]	115 ± 8.03[c]	20.7 ± 7.61[ab]	74.6 ± 7.71[b]
Fructose + Ellagitannins	107 ± 5.43[b]	100 ± 7.45[b]	16.1 ± 2.12[b]	66.9 ± 5.42[b]

Data are presented in "average ± SD" format of six animals. For each parameter, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

4.4 Serum insulin and liver glycogen levels of animals

The serum insulin and liver glycogen levels of the different animal groups are presented in **Table 4.2**. Serum insulin was notably increased by prolonged fructose consumption, although the increase was slightly suppressed by the treatment with the ellagitannins-rich fraction. Similarly, prolonged fructose consumption caused significant ($p < 0.05$) increase in hepatic glycogen content, which was slightly decreased upon treatment with the ellagitannins-rich fraction (**Table 4.2**).

4.5 Homeostasis model assessment of insulin resistance (HOMA-IR) data of animals

The HOMA-IR data of the different animal groups are presented in **Figure 4.6**. The HOMA-IR index significantly ($p < 0.05$) increased (from 2.00 to 5.83) following the prolonged consumption of fructose by the rats, suggesting insulin sensitivity was significantly compromised by prolonged fructose consumption. However, although not significantly, the ellagitannins-rich fraction improved (HOMA-IR index = 3.98) the compromised insulin sensitivity of the fructose-fed rats (**Figure 4.6**).

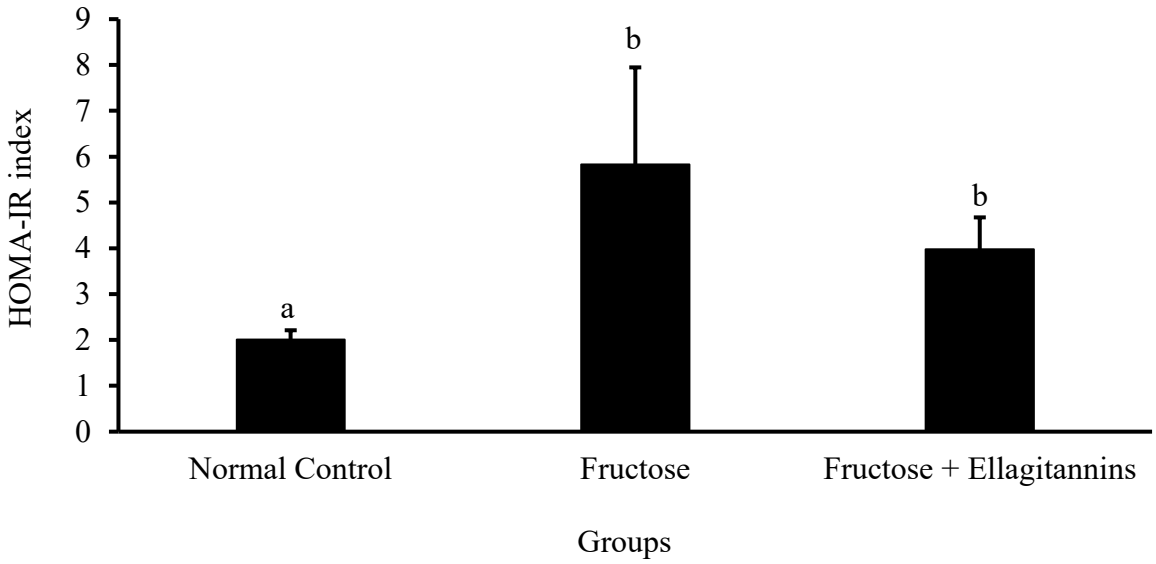


Figure 4.6: HOMA-IR indices of the different animal groups. Data are presented in "average ± SD" format of six animals. The data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

4.6 Hepatic Akt and phosphor-Akt levels of animals

The hepatic phospho-Akt (pSer473) and pan-Akt levels, as well as the hepatic phospho-Akt (pSer473)/pan-Akt ratio of the different animal groups are presented in **Figure 4.7**. While prolonged fructose consumption did not notably alter hepatic pan-Akt level of the rats, it significantly ($p < 0.05$) decreased the level of hepatic phospho-Akt (pSer473).

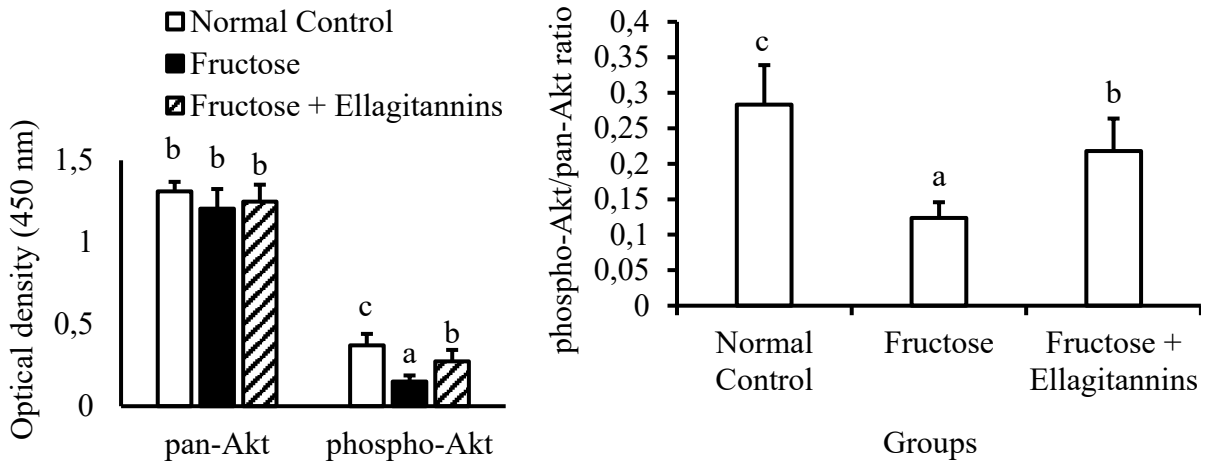


Figure 4.7: ELISA-based Akt phosphorylation in the animals' liver. Data are presented in "average ± SD" format of six animals. For each parameter, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

In coherence with the afore-mentioned, the computed phospho-Akt (pSer473)/pan-Akt ratio was lower in the fructose-fed rats, relative to the rats in the "normal control" group (**Figure 4.7**), reiterating reduced hepatic insulin sensitivity in the fructose-fed rats. Treatment with the ellagitannins-rich fraction significantly ($p < 0.05$) improved hepatic Akt phosphorylation in the fructose fed rats.

4.7 Blood lipids profile of animals

The blood lipids levels of the different animal groups are presented in **Figure 4.8**. Prolonged fructose consumption significantly ($p < 0.05$) increased blood total cholesterol, LDL-cholesterol and triglycerides, while causing a significant decrease in the blood HDL-cholesterol of rats.

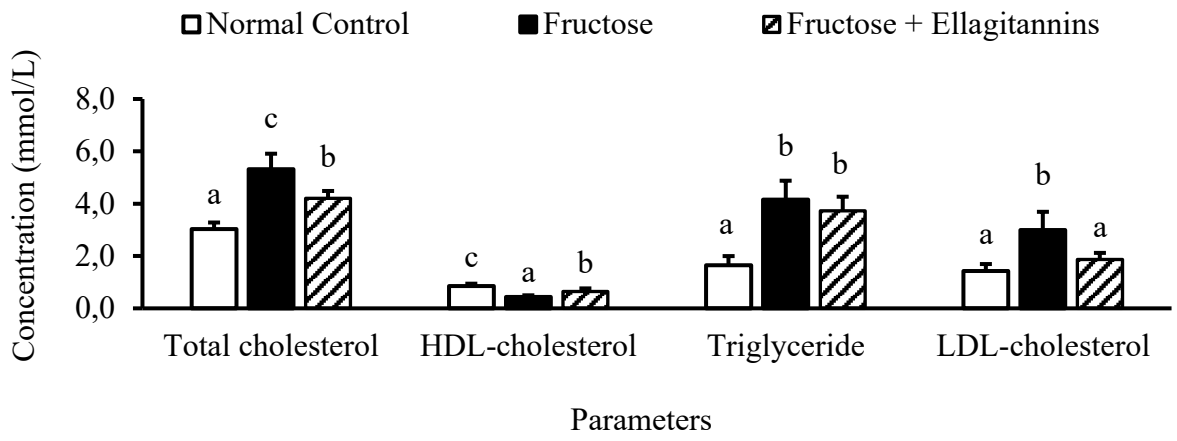


Figure 4.8: Blood lipids profiles of the different animal groups. Data are presented in "average ± SD" format of six animals. For each parameter, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

Treatment with the ellagitannins-rich fraction significantly ($p < 0.05$) suppressed total cholesterol and LDL-cholesterol elevation in the fructose-fed rats (**Figure 4.8**). Conversely and concomitantly, it significantly ($p < 0.05$) suppressed HDL-cholesterol depletion in the fructose-fed rats.

4.8 Hepatic interleukin 1β (IL-1β) level of animals

The hepatic IL-1β level of the rats in the "fructose" group was significantly higher than that of the rats in the "normal control" group (**Figure 4.9**), suggesting that that prolonged fructose

consumption induced hepatic inflammation. However, treatment with the ellagitannins-rich fraction significantly ($p < 0.05$) suppressed the fructose-induced hepatic inflammation.

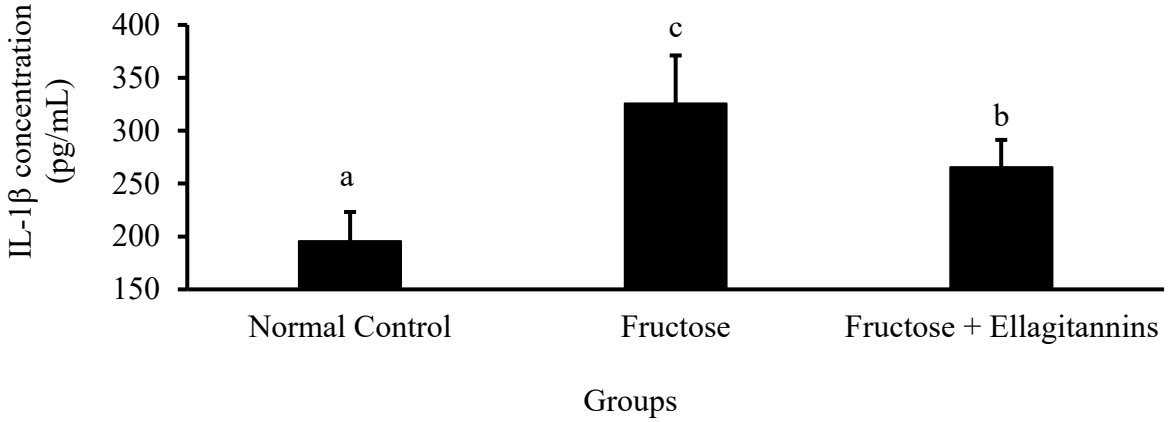


Figure 4.9: Hepatic IL-1β level of the different animal groups. Data are presented in "average ± SD" format of six animals. The data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

4.9 Lipid peroxidation level and antioxidant enzymes activity of animals

The data for the serum and liver lipid peroxidation of the different animal groups is presented in **Figure 4.10**. Prolonged fructose consumption significantly ($p < 0.05$) increased both systemic and hepatic lipid peroxidation in the rats. However, the treatment with the ellagitannins-rich fraction significantly ($p < 0.05$) suppressed hepatic and systemic lipid peroxidation in the fructose-fed rats.

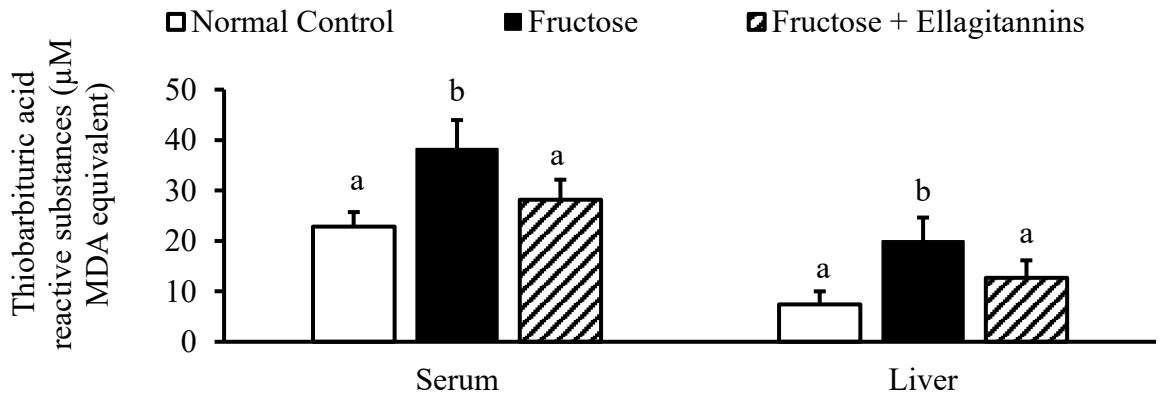


Figure 4.10: Hepatic and systemic lipid peroxidation of animals. Data are presented in "average ± SD" format of six animals. For each parameter, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

The serum and liver catalase activity of the fructose-fed rats dropped following the six weeks consumption of fructose (**Table 4.3**). The "drop" was significant ($p < 0.05$) in the serum. Also, the hepatic superoxide dismutase activity of the fructose-fed rats dropped significantly ($p < 0.05$) following the six weeks consumption of fructose, while the serum catalase activity of the fructose-fed rats was not affected by fructose consumption (**Table 4.3**). Treatment with the ellagitannins-rich fraction significantly ($p < 0.05$) mitigated hepatic antioxidant enzymes depletion in the fructose-fed rats, as suggested by the significantly ($p < 0.05$) higher hepatic catalase and superoxide dismutase activity of the ellagitannins-treated rats, relative to the fructose-fed rats (**Table 4.3**).

Table 4.3: Serum and liver tissue antioxidant enzyme activity

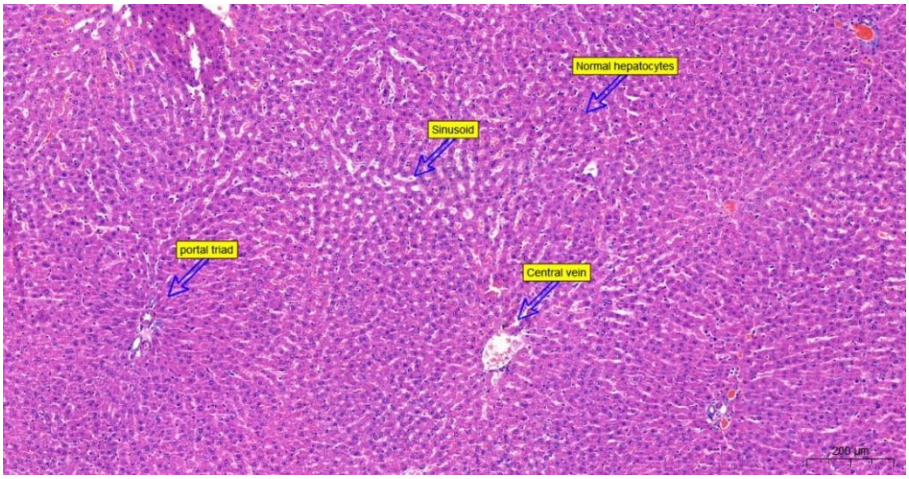
Groups	Catalase activity (U/mg protein)		Superoxide dismutase activity (U/mg protein)	
	Serum	**Liver**	**Serum**	**Liver**
Normal Control	17.1 ± 1.91^{b}	267 ± 20.8^{a}	29.3 ± 6.27	203 ± 38.0^{ab}
Fructose	12.5 ± 3.65^{a}	218 ± 53.6^{a}	29.3 ± 2.27	167 ± 26.1^{a}
Fructose + Ellagitannins	15.4 ± 2.61^{ab}	430 ± 59.8^{b}	28.2 ± 1.75	249 ± 44.1^{b}

Data are presented in "average ± SD" format of six animals. For each parameter, the data between any two treatment groups are significantly ($p < 0.05$) different when there is no common letter assigned to them.

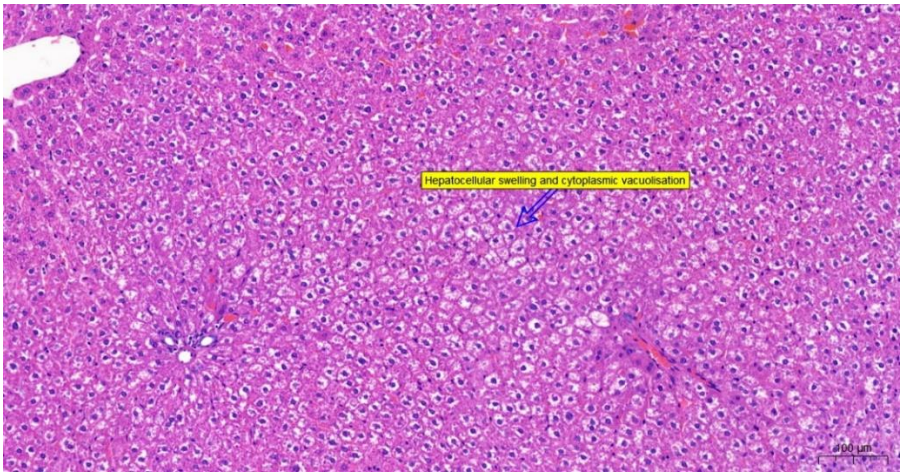
4.10 Hepatic histological data of the different animal groups

Figure 4.11 shows the hepatic histological images and key characteristic features of representative rats randomly selected from the different animal groups. The liver tissue of the animal from the "Normal Control" group depicted normal histological characteristics, such as normal hepatocytes, sinusoid, portal triad, central vein, etc that suggest there is no noteworthy lesions, indicating the liver tissue appears normal. The histology of the liver tissue of the animal from the "Fructose" group showed diffused and moderate cytoplasmic vacuolar change of hepatocytes. The vacuoles were irregular and poorly defined or resembled small variably sized lipid vacuoles in some hepatocytes (**Figure 4.11**), suggesting prolonged consumption of fructose caused vacuolar hepatopathy, possible microvesicular fatty alteration, and glycogen accumulation.

Normal Control



Fructose



Fructose + Ellagitannins

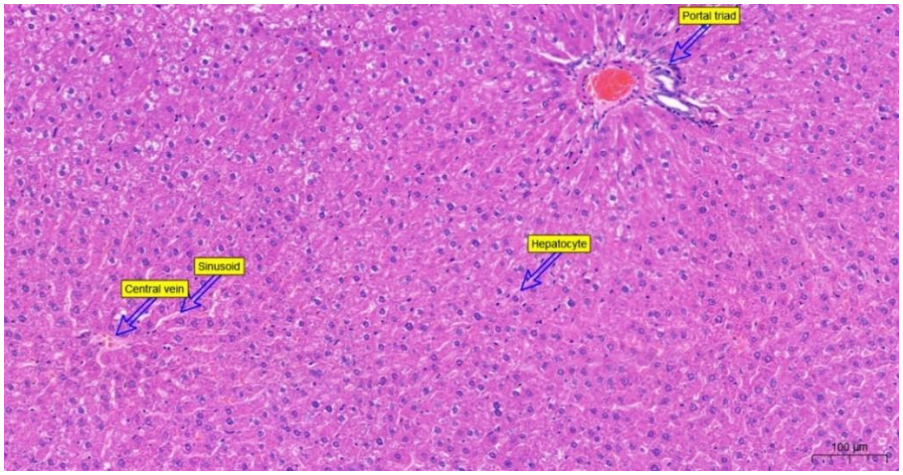


Figure 4.11: Annotated hepatic histological images of rats from the different animal groups.

However, treatment with the ellagitannins-rich fraction appears to mitigate against the fructose-induced vacuolar hepatopathy and possible microvesicular fatty alteration. The liver tissue of the animal from the "Fructose + Ellagitannins" group depicted histological characteristics (normal hepatocytes, sinusoid, portal triad, etc.), thus suggesting absence of notable hepatic lesions (**Figure 4.11**).

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Chronic or prolonged consumption of sugars, including sucrose and fructose has been shown to cause hepatic insulin resistance, proinflammatory responses, increased lipogenesis and visceral adiposity, dyslipidaemia, and increased oxidative stress, which are pathological alterations that promote metabolic syndrome (Wong *et al.*, 2016; Softic *et al.*, 2020). Some studies have shown that pomegranate ellagitannins have potentials to improve metabolic and oxidative health (Ghadimi *et al.*, 2021; Xu *et al.*, 2021; Mashile *et al.*, 2024; Setlhodi *et al.*, 2024). However, the ability of pomegranate ellagitannins to ameliorate sugar-induced metabolic alterations has not been investigated. This study, therefore, reports findings on the ameliorative potential of an ellagitannins-rich fraction from pomegranate peel on fructose-induced metabolic alterations in rats.

Upon consumption, fructose is absorbed in the small intestine mainly via the glucose transporter 5 and mostly metabolised in the liver (Ferraris *et al.*, 2018). Fructose enters the liver via the glucose transporter 2, where it is phosphorylated by fructokinase to form fructose-1 phosphate, and subsequently converted to triose sugars (glyceraldehyde and dihydroxyacetone phosphate) by aldolase B (Tappy, 2012; Regnault *et al.*, 2013). Unlike glucose metabolism (glycolysis), fructose metabolism is not regulated by energy status or ATP levels, leading to an overflow of triose-phosphates. Among other outcomes, this can stimulate fatty acid synthesis via *de novo* lipogenesis and subsequently triglycerides accumulation from fatty acid re-esterification (Tappy, 2012; Regnault *et al.*, 2013). While this may not be detrimental with moderate fructose or sucrose consumption, chronic consumption of these sugars can lead to detrimental weight gain resulting from lipid deposition. Several studies have established the link between prolonged or high fructose or sucrose consumption with detrimental weight gain and the risk of obesity (*et al.*Mansour *et al.*, 2013; Mamikutty *et al.*, 2014; Mahmoud & Elshazly, 2014; Nakagawa & Johnson, 2025).

In the present study, six weeks administration of 20% fructose solution caused significant ($p < 0.05$) weight gain in the rats (**Figure 4.3**). This data is consistent with what was reported in previous animal studies by Mansour *et al.* (2013), Mamikutty *et al.* (2014), Mahmoud and Elshazly, (2014), and Ramos *et al.* (2017), and the findings could be attributed to the stimulation of *de novo* lipogenesis and lipid deposition by the prolonged consumption of

fructose. Furthermore, fructose consumption caused significant ($p < 0.05$) reduction of food intake in the rats (**Figure 4.3**), and this has also been documented in some previous studies (Mamikutty *et al.*, 2014; Ramos *et al.*, 2017). Moreover, the reducing effect of fructose consumption on food intake may be linked to a satiety response and gastric emptying delay. While the effect of fructose administration on food intake and gastric emptying may depend on several factors, such as timing of consumption relative to a meal, the nature (liquid or semisolid) and volume of meal, route of administration and concentration administered (Moran, 2009; Stevens *et al.*, 2009; Evans *et al.*, 2018), it has been shown that fructose consumption or administration stimulates satiety and delay gastric emptying relative to water (Moran, 2009; Stevens *et al.*, 2009). Moreover, there is a link between calorie or food intake and the rate of gastric emptying (Clyburn *et al.*, 2020). Treatment with the ellagitannins-rich fraction did not significantly alter ($p > 0.05$) weight gain or food intake in the fructose-fed rats (**Figure 4.3**).

It was observed that the six weeks consumption of 20% fructose solution significantly ($p < 0.05$) impaired glycaemic control as depicted in the oral glucose tolerance data (**Figures 4.4** and **4.5**). The blood glucose data also showed that the animals in the "Fructose" group had significantly ($p < 0.05$) higher non-fasting and fasting blood glucose levels than the animals in the "Normal Control" group (**Table 4.2**), suggesting that fructose consumption weakened the glycaemic control ability of the animals. The impaired glycaemic control could be attributed fructose-induced insulin resistance in the animals. This is supported by the HOMA-IR data, where the HOMA-IR of the fructose-fed animals was significantly ($p < 0.05$) higher than that of the normal animals (**Figure 4.6**). HOMA-IR is a computed index that is used to determine insulin resistance. Typically, HOMA-IR should not exceed 2.0, except there is insulin resistance (Ader *et al.*, 2014). Hence, the HOMA-IR value of 5.8 in the "Fructose" group denotes insulin resistance.

The serum insulin data also suggests that fructose consumption caused impaired insulin sensitivity (**Table 4.2)**. The animals in the "Fructose" group had a significantly ($p < 0.05$) higher serum insulin concentration, relative to the animals in the "Normal Control" group. The animals in the "Fructose" group concomitantly had a significantly ($p < 0.05$) higher blood concentration, when compared with the animals in the "Normal Control" (**Table 4.2**). The data suggests that insulin-mediated glycaemic control was impaired by fructose consumption, which resulted in increased insulin secretion to compensate for the impaired insulin action (Thomas *et al.*, 2019).

Notably, fructose consumption significantly ($p < 0.05$) reduced hepatic Akt phosphorylation (**Figure 4.7**), which further demonstrates that fructose consumption caused hepatic insulin resistance. This is because Akt phosphorylation is an important step in insulin-mediated glycaemic control and carbohydrate metabolism (Titchenell *et al.*, 2017). Typically, hepatic insulin signalling is initiated by the binding of insulin to insulin receptors on hepatocytes. This binding causes the phosphorylative activation of IRS and PI3K/Akt pathways, which subsequently signals the regulation of glucose and lipid metabolism (Titchenell *et al.*, 2017). Among other effects, hepatic insulin signalling results in the inhibition of circulating glucose production (gluconeogenesis) (Titchenell *et al.*, 2017). Hence, the fructose-induced hepatic insulin resistance may partly be a contributory factor to the observed significantly ($p < 0.05$) higher glycogen content of the animals in the "Fructose" group compared to the animals in the "Normal Control" group (**Table 4.2**). Furthermore, unregulated fructose metabolism can result in overproduction of triose phosphates. The triose phosphates are channelled into the gluconeogenetic pathway to produces circulating glucose and subsequently stored as glycogen (Tappy, 2012; Regnault *et al.*, 2013). Thus, hepatic insulin resistance can exacerbate the production of circulating glucose due to the impaired inhibitory action of insulin on gluconeogenesis.

Treatment with the ellagitannins-rich fraction improved glucose tolerance (**Figures 4.4** and **4.5)**, insulin resistance (**Figure 4.6**), and hepatic Akt phosphorylation (**Figure 4.7**; $p < 0.05$), but reduced blood glucose levels ($p < 0.05$), serum insulin concentration, and liver glycogen content (**Table 4.2**), which all suggest that the treatment improved glycaemic control in the fructose-fed animals. The observed glycaemic control potential of the fraction may be attributed to its predominant ellagitannins constituents, including punicalagin, corilagin, and ellagic acid glucoside (**Figures 4.1** and **4.2**; **Table 4.2**). Type 2 diabetic patients who received 180 mg of ellagic acid for 8 weeks exhibited significant reductions in blood glucose levels (Ghadimi *et al.*, 2021). Additionally, HepG2 cells treated with ellagic acid showed improvement in high glucose-induced insulin resistance (Ding *et al.*, 2019). A 4-week administration of punicalagin at a dose of 70 µg/kg bw caused notable blood glucose reduction in high-fat-diet fed mice (Atrahimovich *et al.*, 2018). Another study in high-fat-diet and streptozotocin-induced diabetic mice showed that punicalagin dose dependently (100, 150 and 200 mg/kg bw) potentiated glycaemic control by reducing hepatic gluconeogenesis and modulating hepatic PI3K/Akt signalling pathway (Jin *et al.*, 2020). In streptozotocin-induced

diabetic rats, the blood glucose-lowering effect of corilagin treatment (10 and 20 mg/kg bw) was also demonstrated (Nandini & Naik, 2019).

Fructose consumption altered the blood lipid profile of rats, causing dyslipidaemia. It significantly increased total cholesterol, LDL-cholesterol and triglyceride, but significantly reduced HDL-cholesterol (**Figure 4.8**). The dyslipidaemic effect of fructose consumption has been well documented (Stanhope and Havel, 2008; Lê *et al.*, 2009), which has been linked to fructose-induced stimulation of lipogenic pathways and lipid accumulation (Tappy, 2012; Regnault *et al.*, 2013). Treatment with the ellagitannins-rich fraction ameliorated the fructose-induced dyslipidaemia by reducing blood cholesterol ($p < 0.05$), LDL-cholesterol ($p < 0.05$) and triglyceride levels and increasing ($p < 0.05$) blood HDL-cholesterol level (**Figure 4.8**), which can be mainly linked to the predominant ellagitannin (punicalagin) identified in the fraction (**Figures 4.1** and **4.2**; **Table 4.2**). A previous study in mice has shown that punicalagin administration was able to reduce the elevated levels of triglycerides and cholesterol caused by high-fat-diet and streptozotocin (Jin *et al.*, 2020). In individuals with hypertriglyceridemia, a punicalagin-containing supplement was shown to reduce blood LDL-cholesterol and triglyceride levels, while increasing the blood HDL-cholesterol level (Quirós-Fernández *et al.*, 2022). Studies in high-fat-diet/high-carbohydrate-fed animals suggest that punicalagin ameliorates dyslipidaemia by modulating AMPK pathway and fatty acid oxidation signalling in hepatocytes and adipocytes, while suppressing lipogenic signalling and adiposity (Cao *et al.*, 2015; Liu *et al.*, 2021). Additionally, dietary metabolites of punicalagin (urolithins) and ellagic acid has been shown to suppress lipid accumulation and lipogenesis in 3T3-L1 adipocytes and hepatocytes (Kang *et al.*, 2016; Cisneros-Zevallos *et al.*, 2020).

Furthermore, hepatic fructose metabolism has be shown to induce inflammation via modulation of protein tyrosine phosphatase 1B (PTP1) expression, c-Jun N-terminal kinase (JNK)/ nuclear factor kappa B (NF-κB) activation and other downstream pro-inflammatory signalling, which triggers the release of inflammatory cytokines, such as tumour necrosis factor-alpha and interleukin-6 (Regnault *et al.*, 2013). The above can interfere with insulin signalling by JNK-dependent serine phosphorylation of the insulin receptor substrate proteins (IRS-1 and IRS-2) and obstructing tyrosine phosphorylation (Regnault *et al.*, 2013). More so, JNK/NF-κB signalling/pathway is a stress-sensitive pathway that is activated by reactive oxygen species (ROS), among other stimuli like cytokines and FFAs accumulation (Hurrle & Hsu, 2017; Masenga *et al.*, 2023), suggesting that oxidative stress is implicated in the inflammatory response and insulin resistance caused by hepatic metabolism of fructose. In the present study,

the prolonged consumption of 20% fructose solution increased hepatic IL-1β level (**Figure 4.9**). This suggests a fructose-induced inflammation in the liver, which can be linked to the impaired insulin signalling observed in the liver (**Figure 4.7**).

Furthermore, fructose consumption also caused hepatic and systemic oxidative stress, as shown by the higher lipid peroxidation level (**Figure 4.10**) and lower antioxidant enzymes activity (**Table 4.3**) of the animals in the "Fructose" group relative to those in the "Normal Control" group. Further histological examination of the liver revealed that liver of the animals in the "Fructose" group depicted vacuoles that were irregular and poorly defined or resembled small variably sized lipid vacuoles in some hepatocytes, suggesting that prolonged consumption of fructose caused vacuolar hepatopathy, and possible microvesicular fatty alteration (**Figure 4.11**).

Treatment with the ellagitannins-rich fraction reduced the fructose-induced hepatic inflammation as shown by the reduced hepatic IL-1β level. It also mitigated the fructose-induced oxidative stress by improving the antioxidant status of the animals and protected the liver against fructose-induced vacuolar hepatopathy. The anti-inflammatory and antioxidant potential of the fraction may be attributed to its phenolic composition, particularly the predominant ellagitannins. Documented evidence in streptozotocin/high-fat diet induced 2 diabetic mice has shown that punicalagin administration dose-dependently mitigated hepatic and renal inflammation by reducing the expression of biomarkers of inflammatory signalling, including interleukin-1 receptor-associated kinase 4 (IRAK-4), monocyte chemotactic protein 1 (MCP-1), NF-κB, toll-like receptor (TLR), and tumour necrosis factor-alpha (TNF-α) (Jin *et al.*, 2020). Concomitantly, it protected against histological damage to the liver and kidney (Jin *et al.*, 2020). Moreover, the anti-inflammatory, antioxidant, and hepatic tissue protective potentials of ellagic acid have been demonstrated in mice with alcoholic liver disease (Zhao *et al.*, 2021).

5.2 Conclusion

Chronic consumption of added sugars, including sucrose and fructose has been shown to be a risk factor for metabolic and cardiovascular diseases. The present study demonstrated the ameliorative potential of an ellagitannins-rich fraction from pomegranate peel on some detrimental metabolic alterations caused by prolonged and high fructose consumption in rats. These include weight gain, impaired glycaemic control, tissue insulin resistance,

dyslipidaemia, elevated oxidative stress, hepatic inflammation, and vacuolar hepatopathy, which is partly linked to fructose-induced hepatic lipogenesis and lipid accumulation. LC-MS analysis showed that the ellagitannins-rich fraction predominantly contained ellagitannins, with punicalagin being the most predominant. The present study showed that the treatment with the ellagitannins-rich fraction ameliorated most of the fructose induced-metabolic disorders by improving glycaemic control, hepatic Akt phosphorylation, insulin resistance, blood lipid profile, and antioxidant status. The treatment also reduced hepatic inflammation and protected against fructose-induced vacuolar hepatopathy. Documented evidence indicates that the ameliorative effect of the fraction could be link to its constituent ellagitannins, especially abundant punicalagin. Ellagitannins from pomegranate peels may serve as useful dietary polyphenols for the prevention or management of sugar-induced metabolic, cardiovascular, and oxidative disorders.

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APPENDICES

Animal Ethics Approval

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS

Animal Research Ethics Committee

01-Oct-2024

Dear Nomonde NP Mapasa

Project Number: UFS-AED2024/0053

Project Title: Investigating the metabolic and antioxidative pharmacological potential of ellagitannins obtained from pomegranate fruit peel in fructose-fed experimental rats

Department: Biomedical Technology - CUT

You are hereby kindly informed that, the UFS Animal Research Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours sincerely

Prof Walter Janse van Rensburg
Chair: Animal Research Ethics Committee

Animal Research Ethics Committee
University of the Free State
T: +27(0)51 401 3942
205 Nelson Mandela Drive, Park West, Bloemfontein 9301, South Africa
www.ufs.ac.za

Acceptance letter for conference presentation

Dear Nomonde, 13 August 2025

I am pleased to inform you that your abstract:

Title	Ellagitannins from pomegranate peel ameliorates insulin resistance, pro-inflammatory singnalling and oxidative stress linked to fructose-induced metabolic disorders in rats
Paper number	To be confirmed
Presenting author	Nomonde P. Mapasa (Centre for Quality of Health and Living, Faculty of Health and Environmental Sciences, Central University of Technology, Free State, South Africa)
Podium / Poster	Podium

has been accepted for presentation at the 58th Annual South African Society for Basic and Clinical Pharmacology Conference (SASBCP2025).

We are still finalising the programming, and we will inform you of your presentation day and presentation number by 15th August 2025.

All presenters must be registered for the Congress. **Please use this link to register:**
https://pharmacy.ukzn.ac.za/south-african-society-for-basic-and-clinical-pharmacologyconference/

The Protea Hotel – Umhlanga, Durban, South Africa, is the official accommodation provider for the 58th Annual South African Society for Basic and Clinical Pharmacology Conference (SASBCP2025), and we have negotiated discounted rates for SASBCP2025 delegates/participants. Accommodation will be sold on a first-come, first-served basis.
Please book now to avoid disappointment and secure a discounted rate. **Use this link to book and pay for your accommodation**:
https://www.marriott.com/event-reservations/reservationlink.mi?id=1744702315643&key=GRP&guestreslink2=true&app

Your sincerely,

Prof. Frasia Oosthuizen (BPharm, MSc, PhD)
SASBCP2025 Scientific Sub-Committee