POTENTIAL OF NARINGIN IN THE PREVENTION OF SWEETENED ALCOHOL-INDUCED CARDIOMETABOLIC DERANGEMENTS IN ADOLESCENT SPRAGUE-DAWLEY RATS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2025

DECLARATION

I, **Jelani Muhammad**, student number **1332401**, declare that this thesis is my work except otherwise acknowledged and that I contributed adequately towards the research findings published in the articles stated below, which are included in my thesis. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, Republic of South Africa. It has not been submitted before for any degree or examination at any other University. All the experimental procedures used in this thesis were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand **(AESC number:2022/10/02/C).**

Jelani Muhammad

Signed:

On…17th .....day of.... September 2025

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the articles by the student as part of his thesis.

Article 1: Title: Effects of voluntarily consumed sweetened alcohol and naringin on cardiac function in male and female Sprague–Dawley rats.

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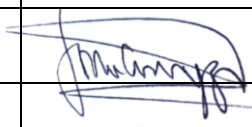
First Author	**Jelani Muhammad**		26 March 2025
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DEDICATION

This research is dedicated to my family for their love, support and encouragement.

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

ORAL PRESENTATIONS

1. **Jelani Muhammad**, Kasimu G Ibrahim, Kennedy Erlwanger, Lebogang Mokotedi. Effects of naringin and sweetened alcohol on cardiometabolic changes in adolescent male and female Sprague-Dawley rats. An oral presentation at the School of Physiology research day, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa on 13th September 2023.
2. **Jelani Muhammad,** Kasimu G Ibrahim, Lebogang Mokotedi, and Kennedy H Erlwanger. The effects of sweetened alcohol and naringin on visceral obesity in adolescent male and female Sprague-Dawley rats. An oral presentation at the Annual General Meeting and Scientific Conference of the Association of Specialist Medical Doctors in Academics, Sokoto-Nigeria, held on 11th January 2024, at the Board room of the College of Health Sciences, Usmanu Danfodiyo University, Sokoto-Nigeria.
3. **Jelani Muhammad,** Kasimu G Ibrahim, Lebogang Mokotedi, and Kennedy H Erlwanger. Naringin protects against sweetened alcohol-induced hepato-steatosis in adolescent male and female Sprague Dawley rats. An oral presentation at the 41st conference of the Physiological Society of Nigeria (PSN) from 12th – 17th February 2024, held at the Department of Human Physiology, College of Medical Sciences, Rivers State University, Port Harcourt - Nigeria.
4. **Jelani Muhammad,** Kasimu G Ibrahim, Lebogang Mokotedi, and Kennedy H

Erlwanger. Sexually Dimorphic effects of sweetened alcohol and naringin on visceral obesity and metabolic health in Sprague Dawley rats. An oral presentation at the 4th Annual General Meeting and scientific conference of the Medical and Dental Specialists Association in Basic Medical Sciences (MeDSABAMS) from 3rd – 6th November 2024, held at the University of Benin-Benin City - Nigeria.

PUBLICATIONS ARISING FROM THIS RESEARCH PROJECT

1. **Jelani Muhammad**, Kennedy H. Erlwanger, Kasimu G. Ibrahim, Lebogang Mokotedi (2024). Effects of voluntarily consumed sweetened alcohol and naringin on cardiac function in male and female Sprague–Dawley rats. *Physiological Reports*, *12*, e70030. https://doi.org/10.14814/phy2.70030.

ABSTRACT

Sweetened alcohol is one of the most consumed alcoholic beverages worldwide, and is gradually getting more popular, especially among adolescents and young adults. The rising consumption of sweetened alcohol necessitates research into the double burden of high sugar and alcohol intake on cardiometabolic health and the development of natural interventions to mitigate these outcomes. Naringin is a flavonoid phytochemical and a key ingredient in grapefruit, tomatoes, and other citrus fruits. It has been associated with various biological benefits, including the prevention and treatment of cardiometabolic diseases. This study aimed to determine the effects of naringin on sweetened alcohol-induced cardiometabolic derangements in adolescent male and female Sprague-Dawley rats.

Eighty, 42-day-old (40 males, 40 females) Sprague-Dawley rats sourced from the Wits Research Animal Facility were randomly allocated to one of four treatment groups, with n = 20 rats in each group (10 males and 10 females), as follows: Control group: received plain gelatine (0.5% fructose in gelatine). Sweetened alcohol (SOH) group: received sweetened alcohol (20% fructose + 10% ethanol) in gelatine. Naringin (NA) group: received naringin (at 50mg/kg/day) in gelatine. Sweetened alcohol + naringin (SOH+NA) group: received sweetened alcohol + naringin (20% fructose + 10% ethanol + 50mg/kg/day naringin) in gelatine. The rats were freely allowed to eat all the treatments made available in wide-mouth glass containers and were given once daily at 10ml per 100g body weight for ten weeks. Body mass was recorded daily, food and water intake were recorded weekly, visceral fat mass, fasting blood glucose (FBG), serum triglyceride, and serum insulin, were measured at termination to assess different metabolic derangements. Serum neutrophil-gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1), were also measured to assess kidney function.

Cardiac function (left ventricular systolic and diastolic function) was assessed using echocardiography, along with blood pressure measurements to assess hypertension, as well as serum cardiac troponin-I concentrations, to assess myocyte damage. Histology of the liver, kidney, and heart using H&E stain was performed to determine hepatic steatosis in the liver, nephropathy indicators including glomerular urinary space, renal corpuscular area, glomerular tuft area, and glomerular density in the kidney, and left ventricular cardiomyocyte diameters in the heart and Picrosirius red stain to determine collagen deposition (collagen area fraction) in the heart.

Rats on sweetened alcohol had decreased ($p<0.05$) food intake (standard rat chow) in both sexes. Male rats that consumed sweetened alcohol had increased ($p<0.05$) terminal body mass, while female rats that consumed sweetened alcohol had increased ($p<0.05$) visceral fat mass.

Sweetened alcohol caused concentric remodelling, impaired left ventricular relaxation, and increased filling pressures in females ($p<0.05$). Male and female rats that consumed sweetened alcohol had increased ($p<0.05$) hepatic steatosis (fatty liver). In male rats, sweetened alcohol led to decreased ($p<0.05$) serum NGAL, KIM-1, and glomerular urinary space, while in female rats, consumption of sweetened alcohol led to decreased ($p<0.05$) serum NGAL, increased ($p<0.05$) KIM-1 concentration, and increased ($p<0.05$) glomerular density (early features of nephropathy). Naringin supplementation had no effects on food intake, body mass, visceral fat mass, or general cardiometabolic health in both male and female rats ($p>0.05$). However, naringin improved ($p<0.05$) concentric remodelling and decreased ($p<0.05$) serum TG, serum NGAL, and KIM-1 in females, while in males naringin caused decreased ($p<0.05$) serum NGAL and increased ($p<0.05$) serum KIM-1 concentrations. When

supplemented with sweetened alcohol, naringin led to decreased ($p<0.05$) serum insulin, increased ($p<0.05$) FBG, increased ($p<0.05$) glomerular density, and increased serum ($p<0.05$) KIM-1 in females, while in males, it led to increased ($p<0.05$) serum KIM-1 and NGAL concentrations.

This study highlighted sexually dimorphic effects of sweetened alcohol consumption and naringin supplementation on cardiometabolic derangements. Female rats were more prone to sweetened alcohol-induced cardiometabolic derangements. While naringin supplementation has the potential to ameliorate some cardiometabolic dysfunctions. Naringin may negatively affect kidney functions when supplemented with sweetened alcohol. Further research will be necessary to evaluate molecular mechanism through which naringin supplemented with sweetened alcohol may negatively affect the kidney.

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8-OHdG	8-hydroxy-2-deoxyguanosine
A	Late filling pressure
a'	Late diastolic velocity
ACC	Acetyl CoA Carboxylase
AChE	Acetylcholinesterase E
AD	Alzheimer's Disease
ALDH	Acetaldehyde dehydrogenase
ADH	Alcohol dehydrogenase
ALD	Alcoholic Liver Disease
AMPK-α	Adenosine Monophosphate-activated Protein Kinase-α
ANOVA	Analysis of variance
ApoB	Apolipoprotein B
ALT	Alanine Transaminase
AREC	Animal Research Ethics Committee
AESC	Animal Ethics Screening Committee
ARRIVE	Animal Research Reporting of *In Vivo* Experiments
AST	Aspartate Transaminase
AUD	Alcohol Use Disorder
BCCAO	Bilateral Common Carotid Artery Occlusion
BM	Body Mass
BMI	Body Mass Index

BP	Blood Pressure
CA	Corpuscular Area
ChREBP	Carbohydrate Response Element-Binding Protein
CLD	Chronic liver disease
CMD(s)	Cardiometabolic Diseases
COX-2	*Cyclooxygenase-2*
CPT-1	Carnitine Palmitoyl Transferase-1
CRP	C-Reactive Protein
CVA	Cardiovascular Accident
CVD	Cardiovascular Diseases
CYP2E1	Cytochrome P450 2E1
DBP	Diastolic Blood Pressure
DGAT2	Diacylglycerol/acyl-CoA Acyltransferase-2
DNA	Deoxyribonucleic acid
E	Trans-mitral blood flow velocity during early diastole
e'	Peak tissue lengthening velocity of the lateral mitral annulus during early diastole
e'/a'	Ratio of early to late mitral annular diastolic tissue lengthening velocity
E/e'	Left ventricular filling pressure
E/A	Ratio of early to late diastolic filling velocity
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal Related Kinase
eNOS	endothelial Nitric Oxide Synthase

FAS	Fatty Acid Synthase
FBG/FPG	Fasting Blood Glucose/Fasting Plasma Glucose
Fe-NTA	Ferric Nitrilotriacetate
FFA	Free Fatty Acid
GAT-1	Glycerol-3-Phosphate Acyltransferase 1
GFR	Glomerular Filtration Rate
GIT	Gastrointestinal Tract
GTA	Glomerular Tuft Area
GLUT2	Glucose Transporter-2
GPx	Glutathione peroxidase
(GPAT-1	Glycerol-3-Phosphate Acyltransferase 1
GST	Glutathione S-Transferase
GUS	Glomerular Urinary Space
HbA1c	Glycated Haemoglobin
Hb	Haemoglobin
HDL	High-Density Lipoprotein
HDL-c	High-Density Lipoprotein-cholesterol
H&E	Haematoxylin and Eosin
HIF1-α	hypoxia-inducible factor subunit alpha
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Hpc2	Human Polycomb protein 2
IDF	International Diabetic Federation
IMPRI	Integrated Molecular Physiology Research Initiative (IMPRI), School of Physiology

IL-6/IL-8	Interleukin-6/8
iNOS	Induced-nitric oxide synthase
IOM	Institute of Medicine
IQR	Interquartile range
JNC	Joint National Committee
JNK	c-Jun N-terminal Kinase
KHK-C	ketohexokinase-C
KIM-1	Kidney Injury Molecule-1
LDL	Low-Density Lipoprotein
LDLPR	low-density lipoprotein receptor
LMIC	low- and middle-income countries
LPS	Liposaccharides
LXA4	Lipoxin A4
LV	Left Ventricle
MAFLD	Metabolic dysfunction-associated fatty liver disease
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MCHC	Mean Corpuscular Haemoglobin Concentration
MetS	Metabolic Syndrome
n	Number
NA	Naringin
Na^+-K^+ ATPase	sodium-potassium ATPase
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dehydrogenase

NAFLD	Non-Alcoholic Fatty Liver Disease
NCD	Non-communicable Diseases
NFκB	Nuclear Factor Kappa-β
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NIH	National Institutes of Health
NO	Nitric oxide
NOS	nitric oxide synthase
NOAEL	Naringin's Oral no Observed Adverse Effect Level
Nrf2	Nuclear Factor-erythroid 2-related Factor
nSREBP-1C	Nuclear SREBP 1-c
OS	Oxidative Stress
PAS	Periodic Acid-Schiff
PCSK9	Protein convertase subtilisin/kexin type 9
PCV	Packed Cell Volume
PPAR-α	Peroxisome Proliferator-activated Receptor alpha
PGC 1-α	peroxisome proliferator-activated receptor-gamma coactivator 1-α
PKG	protein kinase G
PVC tubes	Polyvinyl chloride tubes
RAAS	Renin-Angiotensin-Aldosterone System
RAGE/NF-κB	Receptor for Advanced Glycation End-products/ Nuclear Factor-kB
ROS	Reactive Oxygen Species
SAS	Statistical software for social sciences
SBP	Systolic Blood Pressure

SD	Standard deviation
SD-Rats	Sprague-Dawley rats
SEM	Standard error of the mean
SNS	Sympathetic Nervous System
SOD	Superoxide Dismutase
SOH	Sweetened Alcohol
SOH+NA	Sweetened alcohol + naringin
SREBP	Sterol regulatory element-binding protein
TBARS	Thiobarbituric Acid Reactive Substances
TDI	Tissue Doppler Imaging
TG	Triglyceride
T2DM	Type-2 Diabetes mellitus
TGF-β	transforming growth factor-β
UCP-2	Uncoupling Protein-2
US(A)	Urinary Space (Area)
VLDL	Very low-density lipoprotein
RAGE/NF-Kβ	receptor for advanced glycation end-products/ nuclear factor-kB
T2DM	Type-2 Diabetes Mellitus
TNF-α	Tumour Necrosis Factor-alpha
WGA	Wheat Germ Agglutinin
WRAF	Wits Research Animal Facility
WHO	World Health Organization

CHAPTER ONE – BACKGROUND AND STUDY RATIONALE

1.1 Thesis structure

Seven main chapters plus an appendix section make up this thesis, which is organised in a divided block structure as per the University of the Witwatersrand Faculty of Health Sciences style guide for theses, dissertations and research reports (Style guide for theses, dissertations and research reports, 2016). A list of all the references cited in each chapter is provided at the end of the main chapters and is designated as Chapter Eight.

Chapter one describes the structure and organisation of the thesis and provides an overview of the research problem, which generally covers adolescents' life transition and challenges, sweetened alcohol consumption in adolescents, and its relationship to inappropriate or heavy alcohol consumption, and how it is related to cardiometabolic dysfunction. The public health concern associated with sweetened alcohol and heavy alcohol consumption is also highlighted in this chapter. The healthcare and public health challenges related to the management of fructose- and alcohol-induced cardiometabolic diseases, with the need for alternative preventive and therapeutic strategic measures using affordable medicinal plant-derived phytochemicals, are also discussed. The chapter concludes with the thesis justification, aims and objectives, as well as hypotheses.

Chapter two offers a comprehensive evaluation of the literature pertinent to this study. These include definition, prevalence, risk factors, components, complications, and pathophysiology associated with cardiometabolic dysfunctions/diseases, not only in adolescents but also in adulthood. The chapter also discusses the role of sweetened alcohol (fructose + alcohol) in the pathogenesis of cardiometabolic diseases. The shortcomings of the currently available treatment choices and the need for safe,

accessible, and innovative methods of managing sweetened alcohol-induced cardiometabolic dysfunctions with plant-derived phytochemicals are highlighted as well. In addition, the chapter also discusses the potential benefits of naringin, an abundant phytochemical derived from grapefruits and other citrus fruits with different preventive and therapeutic potentials associated with different cardiometabolic variables.

Chapters three, four, five, and six present the outcomes of the study. Each of these chapters is organised with the following primary sections: introduction, materials and methods, results, discussion, and conclusion. Chapter three has been published in a peer-reviewed journal, while chapters four, five, and six have neither been published nor submitted for publication.

In chapter three, the effects of voluntary consumption of sweetened alcohol and naringin on cardiac function in male and female Sprague-Dawley rats were investigated. The cardiac function of the rats was assessed using echocardiography, blood pressure measurements, and a histological study of the left ventricular cardiomyocyte diameter and fibrosis. The effects of the interventions on body mass and visceral fat mass were also assessed. The outcomes from this chapter were published in a peer-reviewed journal: *Physiological Reports.*

Chapter four focuses on the development of metabolic-associated fatty liver disease (MAFLD), visceral obesity, and other metabolic dysfunctions in male and female Sprague- Dawley rats following voluntary consumption of sweetened alcohol. Specifically, feed and water intake, liver mass, histological determination of hepatic steatosis, quantification of visceral fat mass, and measurement of circulating metabolic parameters (fasting blood glucose, serum triglyceride, and serum insulin) were

reported. The potential preventive and therapeutic effects of naringin on these parameters were also determined.

In chapter five, I investigated and presented the possible protective effect of oral naringin supplementation against the possible development of nephropathy in male and female Sprague-Dawley rats voluntarily consuming sweetened alcohol. The measurements of nephropathy include the determination of kidney mass, kidney histomorphometry, circulating kidney injury molecule-1, and neutrophil-gelatinase-associated lipocalin.

Chapter six is an addendum that contains relevant and supplementary data gathered from the study that was left out of the other chapters. Therefore, in this chapter, the impact of naringin supplementation on further cardiometabolic health metrics of the male and female Sprague-Dawley rats given sweetened alcohol is presented. Red blood cell indices, serum cardiac troponin-I, visceral organ morphometry of the gastrointestinal tract (GIT), pancreatic and testicular masses are covered in this chapter.

Chapter seven summarises the main findings, points out the study's limitations, and makes recommendations for future research. Important conclusions are also made in this chapter.

In chapter eight, all the references cited in all the chapters were listed accordingly.

The appendices section provides the plagiarism declaration form, the ethical clearance certificate, the certificates for ethics modification and extension, and a copy of the published manuscript.

1.2 Background

The American Academy of Paediatrics defines adolescence as an age range between 11 and 21 years (Hagan, 2017). It is characterised as a time for psychological transition matched by increased feelings of self-sufficiency, reduced acceptance of inputs from the elderly, and reduced self-regulatory skills (Haynos & O'Donohue, 2012). Adolescence is a critical period that determines the physical and cognitive development of an individual later in life. It is often during this period that people first encounter lifestyle risk factors for disease, some of which may last into adulthood (de Freitas *et al.,* 2020). Accordingly, what an individual consumes at that age is also important in determining his/her health and well-being in adulthood (Tzioumis & Adair, 2014). In particular, consuming too much of an unhealthy diet (such as alcohol consumption and fructose-rich diets) early in life can make significant negative contributions to their health and well-being later in life, which may lead to the development of cardiometabolic disorders in adulthood (Lelis *et al.,* 2020; Vaschillo *et al.,* 2018).

Hormonal changes during adolescence are believed to affect adolescents' sensitivity to the effects of alcohol, making them less sensitive to its negative effects and causing increased consumption (Tapia-Rojas *et al.,* 2017). This can lead adolescents to engage in binge drinking, which can adversely affect the functions of their vital organs (Ojeda *et al.,* 2022). Therefore, alcohol consumption among adolescents is not without consequences to their health and well-being, as the long-term effects of alcohol consumption have indeed been linked to the development of cardiometabolic derangements (Nogales *et al*., 2014; Yan *et al.,* 2018), renal (Binder *et al.,* 2016) and liver (Sobrino *et al.,* 2019) impairments.

Alcohol consumption in Africa is ranked as the second highest in the world and is particularly high among adolescents (Peacock *et al.,* 2018). Sadly, about 3.3% of all deaths in Africa are attributed to alcohol consumption, which also accounts for 2.4% of all disability-adjusted life years (DALYs) (Hammer *et al*., 2018). Alcohol is a leading risk factor causing mortality and disability in sub-Saharan Africa (Lim *et al.,* 2012). In South Africa, according to research utilising nationally representative survey data, roughly 50% of South Africa's male population and 20% of its female population drink alcohol (Fontes Marx *et al.,* 2021), with about 32% of females and 48% of males engaged in unhealthy alcohol consumption (Vellios & van Walbeek, 2018). There are cultural and lifestyle factors that are reported to influence increased alcohol consumption among adolescents in South Africa. Some of these factors include poor self-esteem that increases the vulnerability to peer pressure, poor parent-child relationships, weak or inadequate parental attention, dating partner's influence, social media advertisement, emulating celebrity lifestyles, and higher education transition, which introduces freedoms not experienced whilst under parental/guardian care (Hlomani-Nyawasha *et al*., 2020). (Hlomani-Nyawasha *et al*., 2020). Consequently, the WHO placed South Africa among the top countries with a high prevalence of alcohol consumption (Hammer *et al*., 2018).

Given the high levels of alcohol consumption, it is not surprising that alcohol has been identified as one of the four main risk factors for the increased prevalence of cardiometabolic diseases (CMDs) – which refers to the main cardiovascular disease risk factors, they include insulin resistance, obesity, and high blood pressure, and fatty liver (Guan *et al*., 2022), with the other three modifiable risk factors being smoking, lack of physical activity, and eating unhealthy diets (WHO, 2022), such as increased sugar/fructose consumption. Intake of simple sugars, particularly added fructose, has

sharply increased in recent decades, partly as a result of the considerable rise in consumption of sugar-sweetened beverages (SSB) in which fructose is the popular sweetener in both carbonated and non-carbonated drinks (Joh *et al*., 2021). Adolescents, as compared to other age groups, consume the most SSBs, accounting for about 10% of daily calories (Rosinger *et al*., 2017). Additionally, consumption of SSB is rising quickly worldwide, especially in developing nations (Malik & Hu, 2019). It has been reported that in five years, more than 50% of adolescents in 53 low- and middle-income countries would consume carbonated soft drinks at least once daily (Yang *et al*., 2017). Due to its sweetening qualities, fructose is frequently utilised in beverages, dairy goods and processed pastries. Fructose is directly blended with alcohol to produce sweetened alcoholic beverages, which enhances the palatability of the beverages (Wakabayashi *et al.,* 2021). Adolescents whose bodies are going through important periods of development are one of the main populations that consume sweet products (Heiss *et al*., 2022), including sweetened alcoholic beverages (Binakonsky *et al.,* 2011; Crews, 2015; Siegel *et al.,* 2016). Common sweetened alcoholic beverages found in South Africa include ciders, pre-mixed drinks, cream liqueurs (e.g. Amarula), sweet wines, cocktails and shooters, and flavoured alcoholic beverages.

Consuming fructose has a strong link to the emergence of CMDs (Lelis *et al*., 2020). Fructose diminishes feelings of fullness, promotes ectopic fat storage, raises adipogenesis, boosts oxidative stress, decreases insulin sensitivity, and causes vascular damage, thus linking increased fructose consumption to the emergence of CMDs (Lelis *et al.,* 2020). Increased waist circumference, fasting blood glucose (FBG) concentration, raised blood pressure, increased serum triglycerides, and increased serum cholesterol have been associated with high fructose consumption (Jung *et al*., 2022). A high intake of drinks with added fructose raised cardiovascular disease (CVD)

risk by more than 25% (Malik *et al*., 2010). Poor diet (such as excess fructose consumption), sedentary lifestyle and obesity are modifiable risk factors also linked to cardiovascular diseases (WHO, 2022). Fructose consumption has been related to cardiac inflammation and cardiac fibrosis by facilitating the release of proinflammatory mediators and increasing oxidative stress (Kang *et al*., 2016).

On an individual basis, according to different research reports, increased alcohol and fructose consumption have a negative consequence on adolescent health. Combining them as sweetened alcoholic beverages presents a double simultaneous burden of increased sugar and increased alcohol consumption.

Sweetened alcohol is an alcoholic beverage consisting of liquor combined with a sweetener (Wakabayashi *et al*., 2021). Commonly, alcohol is combined with sweetened gelatine and referred to as ‘jello-shots’ or ‘gelatin-shots’, with different types of ‘jello-shots’ available in the market (Crews, 2015). Consumption of these gelatine shots is problematic as many people do not consider them as an alcoholic beverage, and as such are unaware of the potential danger associated with them (Crews, 2015). This is partly because the taste resembles that of the sweetener that is combined with it, rather than the actual taste of alcohol. As a result, a large amount can be consumed within a short period, causing more adverse effects than conventional alcohol, as this can lead to binge drinking (Binakonsky *et al.,* 2011; Albers *et al.,* 2015; Siegel *et al.,* 2016; Wakabayashi *et al*., 2021). The sweet taste also entices adolescents to start consuming alcohol at a younger age than their counterparts who drink unsweetened beverages (Crews, 2015).

It has been reported that people who for the first time try to taste alcohol are more likely to fall victim to alcohol consumption when they start with a combination that gives a sweet and flavoured taste that masks the unpleasant taste of conventional alcohol

(Copeland *et al*., 2007). Excess alcohol and sugar (sweeteners) consumption is not without consequences, as several disease conditions are linked to excess alcohol and sugar consumption, with CMDs in the lead (Fagherazzi *et al*., 2013).

1.3 Alcohol, fructose, and cardiometabolic diseases

There is an increase in the consumption of alcohol and sugar-sweetened beverages globally (Vorster *et al*., 2014). In South Africa, studies have shown that consumption of sugar-sweetened beverages is on the increase, with an accompanying rise in risk factors for non-communicable diseases in both rural and urban areas (Vorster *et al*., 2014; Manyema *et al*., 2016). Excessive sugar and alcohol consumption are poor dietary habits that are implicated among the risk factors for the onset of CMDs (Fagherazzi *et al.,* 2013; Herman & Birnbaum, 2021; Lelis *et al*., 2020).

Increased fructose intake leads to continuous hepatic fructose absorption and metabolism, mediated by glucose transporter-2 (GLUT2) and ketohexokinase-C (KHK-C) (Herman & Birnbaum, 2021). This causes intrahepatic lipid accumulation, dyslipidaemia, decreased insulin sensitivity, and hyperuricaemia (Herman & Birnbaum, 2021). In response to cardiac stress caused by excessive fructose consumption, hypoxia-inducible factor subunit alpha (HIF1-α)-driven stimulation of fructose absorption and metabolism in the heart promotes anabolic cardiac development as well as systolic and diastolic dysfunction (Herman & Birnbaum, 2021).

There is a positive relationship between high sugar consumption and the development of obesity and metabolic syndrome, as evidenced by increased levels of very low density lipoprotein (VLDL), glucose intolerance, insulin resistance, and accumulation of hepatic lipids (de Oliveira *et al*., 2020). Some of the mechanisms that link increased fructose consumption to liver diseases include oxidative stress and inflammation. The inflammatory signalling pathways and lipogenesis are activated, leading to fibrosis,

cirrhosis, and hepatocellular carcinoma (Muriel *et al*., 2021). Studies in clinical, experimental, and epidemiological settings have demonstrated that fructose can have a number of harmful effects when consumed in excess of what is advised. Higher fructose intake promotes a positive energy balance by reducing satiety, increasing adipogenesis, which results in visceral fat accumulation, and causing ectopic fat formation, particularly in the liver and skeletal muscle, which results in vascular damage brought on by insulin resistance, inflammation, and impaired lipid metabolism, which raise arterial blood pressure increasing the risk of cardiovascular and metabolic diseases (Lelis *et al*., 2020).

Just as fructose consumption contributes to CMDs, alcohol consumption has also been associated with an increased risk of CMDs. This section will, therefore, mainly focus on risks of excessive alcohol consumption, especially in the context of adolescents' health. Notably, some research findings have linked mild to moderate alcohol consumption to offer some protection and reduced risks against metabolic syndrome (Sun *et al*., 2014), coronary heart disease, and stroke (Single *et al*., 1999). However, high alcohol consumption is linked with some cardiovascular conditions such as hypertension, arrhythmias, and heart failure (Hietanen *et al*., 2020), and metabolic syndrome (Husain *et al*., 2014; Sun *et al*., 2014; Wang *et al.,* 2015). In an animal study, it was shown that alcohol consumption by adolescent rats altered the functional growth of the heart and resulted in 'pathological hypertrophy' that persisted even after the withdrawal of alcohol (Ai *et al*., 2020). The study also reported that adolescent rat hearts are more prone to damage after alcohol exposure compared to the control (Ai *et al*., 2020). Heavy alcohol consumption is physiologically believed to be associated with increased clotting and reduced ventricular fibrillation threshold (Rehm *et al*., 2010). Apart from other health conditions related to alcohol consumption,

the risk of chronic diseases such as coronary heart disease and sudden heart attack has also been linked to the pattern of alcohol consumption (Hietanen *et al*., 2020).

Alcohol consumption is one of the major causes of liver disease and liver damage (Parker *et al*., 2019). The majority of individuals who drink alcohol frequently develop hepatic steatosis that is usually mild and silent, but it is typically regarded as a precursor to more severe forms of liver disease (Parker *et al*., 2019). About one-third of patients with steatosis

progress to steatohepatitis and almost 10 in every 100 develop cirrhosis (Hughes *et al*., 2019). Almost half of all liver cirrhosis cases are alcoholic cirrhosis globally, associated with significant mortality, causing about 10% of all deaths related to alcohol, and about half of all alcohol-related deaths are associated with liver disease, and this almost causes 22 million DALYs annually (Rehm *et al*., 2013; Peacock *et al*., 2018; Lucey, 2019). Recurrent inflammation and oxidative stress result in liver fibrosis due to irregularities between synthesis and degradation of the extracellular matrix, resulting in chronic hepatic injury from alcohol consumption (Hernández-Aquino & Muriel, 2018). Liver fibrosis leads to cirrhosis, which destroys the liver parenchymal architecture, causing insufficient blood supply to the hepatocytes and leading to liver failure (Ramachandran & Iredale, 2009) and hepatocellular carcinoma (Serfaty & Lemoine, 2008).

1.4 Management of cardiometabolic diseases and challenges

Despite remarkable advancements in global health care, a statement from the 2009 Institute of Medicine (IOM), National Institutes of Health (NIH), USA Summit on Integrative Medicine and Health of the Public stated that “The disease-driven approach to care has resulted in spiralling costs as well as a fragmented health system that is reactive and episodic as well as inefficient and impersonal.” (Rao, 2018). The statement

confirmed the fact that despite the progress made in the treatment of diseases, the incidence and prevalence of these diseases are still on the increase. Despite the remarkable progress made in the modern approaches to the management of CMDs, there is an increasing burden and prevalence of cardiovascular diseases, diabetes, hypertension, and obesity (Akik *et al*., 2025).In the past few decades, both the prevalence of type 2 diabetes and obesity rates have significantly increased (Sattar *et al*., 2020). A collection of biochemical and clinical abnormalities known as metabolic syndrome (MS) raises the risk of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (Charles-Davies & Ajayi, 2023). It is a major global clinical and public health concern that is becoming more common. Worldwide, MS affects more than one billion individuals (Charles-Davies & Ajayi, 2023). Its incidence varies among populations in Africa and can reach 50% or higher. MS has a prevalence of about 42% in South Africa (Jean-Luc Gradidge & Crowther, 2017). This is an indication that challenges do exist that need an integrative approach to a more affordable, result-oriented, and disease-focused model of disease treatment and prevention. For many decades, traditional medicine practitioners across the globe have used herbal medicine and other phytochemicals and experienced the benefits of their therapeutic characteristics (Rao, 2018). Usually, several classes of medications and different treatment modalities are required to manage CMDs in an afflicted individual. Examples of different medications used in pharmacotherapeutic approaches include antihypertensives for blood pressure regulation, lipid lowering agents such as statins to manage dyslipidaemia, antidiabetic agents like sulfonyl ureas for control of diabetes mellitus, GLP-1 receptor agonists for glucose control and weight loss, and icosapent ethyl that reduces cardiovascular adverse events such as obesity and triglycerides (Antwi *et al*., 2024; Patel *et al*., 2025). Some of these medications are either non-affordable, non-accessible, and/or related to different side effects, which render

compliance very difficult (Jamshidi-kia *et al*., 2018; Rao, 2018; Shi *et al*., 2019). Numerous studies have investigated plant-based dietary patterns, as well as individual foods and components, to determine the role of important nutrients in the prevention, protection, and reversal of cardiometabolic diseases (Mullins & Arjmandi, 2021). The use of phytochemicals as modulators of several cellular pathways to prevent cardiometabolic diseases appears to be gaining popularity, due to their affordability, availability, and with fewer or no side effects (Jamshidi-kia *et al.,* 2018). These phytochemicals work by controlling molecular mechanisms that either interfere with the inflammatory pathways, such as by reducing the production of pro-inflammatory cytokines and other modulators, or synergise with the anti-inflammatory pathways, such as by increasing the production of anti-inflammatory cytokines (Nisar *et al*., 2023). Although phytochemicals may help prevent and treat disease, there are a number of drawbacks to take into account. These include possible adverse effects, drug interactions, a lack of standardisation, and difficulties with absorption and bioavailability (Yiwen *et al*., 2024).

With continued research, nutraceuticals, which are products derived from food and food components that have nutritional, medical, and health benefits, including disease treatment and prevention (Puri *et al*., 2022) are projected to play a critical role in delivering well-tested complementary treatments that will have an important role in ensuring affordable healthcare (Rao, 2018). Studies have shown that nutraceuticals may be able to alleviate the growing burden of CMDs, and they are anticipated to lower the enormous costs associated with healthcare, providing comfort to the affected person as well as the relevant authorities (Bruno *et al*., 2022).

In summary, alcohol and fructose are important dietary risk factors in the development of cardiometabolic diseases. Combining them in the form of sweetened alcohol will have a double burden of increased fructose and increased alcohol consumption. Sweetened

alcohol is becoming popular in the modern world, and its increased consumption is believed to be associated with increased risk and prevalence of cardiometabolic diseases, as it is reported to facilitate heavy alcohol consumption, because the sweet taste masks the unpleasant taste of the conventional alcohol and thereby increases its palatability. As of today, because it is a relatively new social/health burden, there are no standard treatment modalities for cardiometabolic derangements associated with sweetened alcohol. The therapeutic and preventive potential of phytochemicals in CMD has been proven to be effective and promising (Rajadurai & Stanely Mainzen Prince, 2006; Malakul & Pengnet, 2018; Park *et al*., 2018; Uryash *et al*., 2021; Akin *et al*., 2022; Shackebaei *et al*., 2023).

1.5 Rationale for the study

The trend of adolescents consuming more sweetened alcoholic beverages may result in a double burden of high alcohol and sugar intake, further increasing the risk of cardiometabolic diseases. Individually, studies have reported the negative consequences of increased sugar and alcohol consumption; however, the combined effects in the form of sweetened alcohol have not been well studied. Although there has been progress in the treatment and management of CMDs, the incidence and prevalence of these diseases in high (Rao, 2018). It is necessary to make efforts on disease prevention because individuals tend to only attend medical facilities when they are ill (Saad *et al*., 2017). One of the best times to prevent CMDs is during adolescence and continues throughout life (Rao, 2018). According to studies, medicinal plant-derived phytochemicals are potential agents in tackling the growing burden of CMDs and are anticipated to lower the enormous healthcare costs, benefiting both the individual and the relevant authorities (Bruno *et al*., 2022).

Even though it has been demonstrated that females consume more sweetened alcohol than males, studies that examine the cardiometabolic effects of sugar/fructose and alcohol intake are typically skewed and concentrate more on males. To address this gap, both male and female Sprague-Dawley rats were used in this study.

While a forced alcohol paradigm has been employed in previous animal studies, in this study, I selected a realistic voluntary alcohol intake model. Multiple medications are necessary for the management of CMD. The medication combinations include antihypertensives, antidiabetics, and lipid-lowering agents. This can result in an inappropriate prescription, poor compliance, over- or underdosing, poor drug selection, inadequate monitoring, adverse drug effects, drug interaction, unnecessary waste, and expenses (Seidu *et al*., 2023). As a result of these challenges, alternatives need to be explored, and one of the best alternatives is the use of phytochemicals due to their affordability, accessibility, and multiple constituent phytochemicals with nutraceutical properties, and often minimal or no adverse effects (Rao, 2018; Saad *et al*., 2017). Therefore, this study investigated the potential preventive and therapeutic effects of naringin supplementation on cardiometabolic dysfunction induced by voluntary sweetened alcohol consumption in male and female adolescent Sprague-Dawley rats.

1.6 Aim and Objectives

1.6.1 Aim

The study aimed to explore the potential of dietary naringin supplementation to protect against the development of cardiometabolic derangements induced by the consumption of sweetened alcohol in adolescent male and female Sprague-Dawley rats.

1.6.2 Specific objectives

The specific objectives of the study were to explore the effects of naringin and sweetened alcohol on:

Cardiac function, by measuring:

a) Blood pressure using the tail-cuff method to assess hypertension as a marker of cardiovascular function

b) Left ventricular systolic and diastolic function using echocardiography to assess cardiac contractility and relaxation.

Metabolic function, by determining:

a) The levels of insulin, glucose, and triglycerides, to assess insulin resistance, diabetes mellitus, and dyslipidaemia as components of cardiometabolic diseases.

b). adiposity: the weight of visceral fat within the abdomen, to assess obesity as an important risk factor for cardiometabolic diseases.

Kidney function, by measuring

a) neutrophil gelatinase-associated lipocalin (NGAL), and Kidney Injury Molecule-1 (KIM- 1) as surrogate indicators and early markers of kidney function, to assess the function of the kidney, as a risk factor and a component of cardiometabolic diseases.

Histology of the heart, liver, and kidney, to determine:

a) cardiomyocyte diameter, and cardiac fibrosis

b) hepatic steatosis

c) nephropathy indicators, including glomerular urinary space, renal corpuscular area, glomerular tuft area, and glomerular density

General cardiometabolic health, by measuring:

a). red blood cell indices, to assess the effects of sweetened alcohol and naringin on red blood cell production

b). cardiac Troponin-I, to further assess the cardiac function

c). morphometry of the GIT and visceral organs, to assess the effects of sweetened alcohol and naringin on these organs

1.7 Hypothesis

H1:

1. Naringin protects against cardiac dysfunction associated with sweetened alcohol consumption in adolescent rats.
2. Naringin protects against general metabolic dysfunction associated with sweetened alcohol consumption in adolescent rats.
3. Naringin protects against metabolic-associated fatty liver disease associated with sweetened alcohol consumption in adolescent rats.
4. Naringin protects against nephropathy and kidney injury associated with sweetened alcohol consumption in adolescent rats.

H0:

1. Naringin does not protect against cardiac dysfunction associated with sweetened alcohol consumption in adolescent rats.
2. Naringin does not protect against general metabolic dysfunction associated with sweetened alcohol consumption in adolescent rats.

3. Naringin does not protect against metabolic-associated fatty liver disease associated with sweetened alcohol consumption in adolescent rats.
4. Naringin does not protect against Nephropathy and kidney injury associated with sweetened alcohol consumption in adolescent rats.

CHAPTER TWO - LITERATURE REVIEW

2.1 Introduction

Alcohol consumption is a significant global health concern (Barbería-Latasa *et al*., 2022). With an estimated 2.3 billion people drinking alcohol worldwide, the social landscape of many societies is strongly influenced by alcohol consumption (WHO, 2021). Despite several national and international attempts to minimise alcohol consumption, the global pattern of alcohol consumption exhibits a growing trajectory and is predicted to continue to increase, producing a tremendous global socioeconomic and health burden. (Liu *et al*., 2023). Alcohol consumption is linked to several acute and long-term health problems, including cardiovascular disease, metabolic diseases, and cancer (Abbas *et al*., 2023).

Alcohol use is also associated with a greater death rate than conditions like diabetes mellitus, HIV/AIDS, and tuberculosis (WHO, 2021). In fact, approximately three million fatalities (5.3% of all deaths) and more than 132 million disability-adjusted life years (DALYs) (about 5% of all DALYs) are estimated to be caused by alcohol consumption annually, globally (WHO, 2021). Additionally, about 13.5% of all deaths occurring among adolescents and young adults are alcohol related (WHO, 2022). In addition to the immediate ill-health effects on the drinker, inappropriate alcohol consumption has a substantial detrimental influence on society as a whole. It is associated with several unfavourable social outcomes, including car crashes, injuries due to accidents while operating machinery when drunk, and family strife, and it places a load on the criminal justice system (Moss, 2013).

For decades, there has been discourse over the potential advantages of low to moderate alcohol intake over abstinence and heavy drinking in terms of cardiometabolic health and mortality (van de Luitgaarden *et al*., 2022). It is believed that an imbalance between the positive and negative effects of alcohol intake

contributes to the burden of diseases related to alcohol (Barbería-Latasa *et al.,* 2022). According to some reports, the safe amount of alcohol consumption should be zero, indicating that no degree of alcohol consumption is of health benefit (Burton & Sheron, 2018), as it has been linked to several disorders that might cause health problems (Bryazka *et al*., 2022).

Indeed, several studies report that alcohol consumption has been negatively linked to an increased risk of developing various indices of cardiometabolic diseases (CMDs) (Piano, 2017; Vaschillo *et al*., 2018; Lankester *et al*., 2021; Wang *et al*., 2022). Thus, the amount of alcohol consumed may indicate a higher or lower risk for specific cardiometabolic variables. Literature has associated mild-to-moderate alcohol consumption with some protection and reduced risks against metabolic syndrome (Sun *et al*., 2014), and some cardiovascular diseases such as coronary heart disease and stroke (Single *et al*., 1999). However, some cardiovascular conditions such as hypertension, arrhythmia, and heart failure (Puddey *et al*., 1999), and metabolic syndrome (Sun *et al.,* 2014) are adversely affected by excess alcohol consumption (Husain *et al.,* 2014). These effects are largely associated with different metabolic processes and mechanisms that occur during alcohol metabolism and that are largely dependent on the amount of alcohol consumed.

The current increase in the prevalence of cardiometabolic disorders has also been associated with the greater availability of sugar, especially in sweetened beverages (Herman & Birnbaum, 2021). Increased consumption of different sugars in food and beverages significantly affects several cardiometabolic risk variables, including glucose intolerance and insulin resistance, hypertension, dyslipidemia, endothelial dysfunction, obesity, inflammation, and adipocyte dysfunction, which are all hallmarks of cardiometabolic diseases (Alberti *et al*., 2009).

Concerning the relationship between sugar and alcohol consumption, it is evident that on an individual basis, increased sugar (Herman & Birnbaum, 2021), and alcohol (Lankester *et al.,* 2021; Barbería-Latasa *et al.,* 2022) consumption is related to increased risk for the development of CMDs. When consumed in the form of flavoured alcoholic beverages, sweetened alcohol, or jello shots, their combined effects may potentially present a double burden of increased risk for the development of cardiometabolic diseases. These flavoured alcoholic beverages, because of their pleasant nature, have been associated with increased or heavy alcohol consumption (Binakonsky *et al.,* 2011; Siegel *et al.,* 2016). This is reflected in recent statistics showing an increasing trend of consumption of sweetened alcoholic beverages, especially by adolescents and young adults (Siegel *et al.,* 2016).

There are limited studies on the impact of sweetened alcohol on different cardiometabolic indices. However, individual effects of alcohol and sugar or fructose has been reported in various studies. The objective of this section is to provide an update on the increased consumption of sweetened alcohol as a lifestyle transition, how sweetened alcohol facilitates heavy or increased consumption of alcohol and sugar/fructose, and the relationship between increased alcohol and sugar/fructose to the increasing burden of cardiometabolic disease. It is hoped that the public will be educated on the impending risks associated with the consumption of sweetened alcoholic beverages as they relate to heavy alcohol consumption.

2.2 Sweetened alcoholic beverages: types and consumption patterns

Sweetened alcohol is an alcoholic beverage that is blended with different types of sweeteners (Crews, 2015; Wakabayashi *et al*., 2016), of which fructose and glucose are the most commonly used, and in varying concentrations (Wakabayashi *et al.,* 2021). There are different types of sweetened alcoholic beverages depending on the

content and preparations (Crews, 2015; Fortunato *et al.,* 2014). Table 2.1 shows the most common types of sweetened alcoholic beverages.

Table 2.1: Common types of sweetened alcoholic beverages

Sweetened alcoholic beverage	Constituents
Jello shot	Vodka, pure grain alcohol, or flavoured liquor in sweetened gelatine
Flavoured malt beverages	Malt beverages and spirits
Premixed cocktail	Spirit and cocktail
Supersized alcopops	Malt, wine, or distilled spirit
Cocktail	Carbonated drink and spirit. E.g. Coke and rum or brandy and coke

Source: (Binakonsky *et al*., 2011; Crews, 2015; Fortunato *et al*., 2014; Siegel *et al*., 2016; Wakabayashi *et al*., 2021).

2.1.1 Common sweeteners in sweetened alcoholic beverages

Sucrose and fructose are common sugars added to beverages and food products. Fructose is usually added in the form of high-fructose corn syrup that is manufactured by an enzymatic process that converts corn starch to its glucose and fructose monosaccharides (Herman & Birnbaum, 2021). The most widely used formulation, which is added to beverages, is composed of 45% glucose and 55% fructose (Wakabayashi *et al.,* 2021).

Other common sweeteners used in alcoholic beverages include:

1. Simple Syrup: A mixture of equal parts sugar and water, often used in cocktails like Daiquiris and Old Fashioneds.

2. Honey Syrup: Made by mixing honey with water, it adds a floral sweetness to drinks like Bee's Knees.
3. Agave Syrup: Derived from agave plants, it's commonly used in tequila-based cocktails.
4. Demerara Syrup: Made from Demerara sugar, it has a rich, caramel-like flavor, perfect for enhancing dark spirits.
5. Fruit Juices: Natural sweeteners like orange juice, pineapple juice, and cranberry juice are often used in cocktails.
6. Grenadine: A sweet, red syrup made from pomegranate juice, used in drinks like Tequila Sunrise.
7. Sugar Alcohols: Such as xylitol, erythritol, and sorbitol, which are used in some low-calorie or sugar-free beverages

Glucose, fructose, and sucrose are just a few of the sugars that are frequently found in sweetened alcoholic beverages (Wakabayashi *et al*., 2021). Furthermore, sugar alcohols such as maltitol, sorbitol, erythritol, Aspartame, Saccharin, and xylitol are commonly utilised, especially in low-calorie or sugar-free products (Abbasi *et al*., 2021; Ruiz-Ojeda *et al*., 2019). Fructose, sucrose and glucose have almost similar metabolic effects, except that glucose has a strong peripheral sensory effect and crosses the blood-brain barrier more easily when compared with fructose and sucrose, and therefore, when combined with alcohol, glucose produces faster and more central nervous system effects compared to fructose and sucrose (Wakabayashi *et al*., 2021). Other sugars (sugar alcohols) used to make sweetened alcoholic beverages provide a similar or sweeter taste compared to fructose, glucose or sucrose. However, it has very low caloric value, and therefore, fewer metabolic effects (Ruiz-Ojeda *et al*., 2019; Wakabayashi *et al*., 2021).

The pleasant taste of these sweeteners encourages overconsumption, which results in a surplus of calories consumed, weight gain, and obesity (Herman & Birnbaum, 2021). This trend is particularly evident in the rising popularity of sweetened alcoholic beverages.

2.1.2 Consumption trends and patterns

Sweetened alcoholic beverages were introduced many decades ago, and since then, there has been an increase in popularity and acceptance globally (Mosher & Johnsson, 2005).

These beverages have been projected to be among the most consumed alcoholic beverages globally (Fortunato *et al.,* 2014). The pattern of alcohol drinking has largely been attributed to the consumption of sugar-sweetened alcoholic beverages (Wakabayashi *et al.,* 2021). It has also been reported that more than 50% of the younger population who are involved in inappropriate alcohol consumption are particularly engaged in the consumption of sweetened alcoholic beverages (Albers *et al.,* 2015). Frequent consumption of sweetened alcohol has been reported among people of all ages (Wakabayashi *et al.,* 2016). Studies have shown that increased consumption of alcohol, especially among younger adults, is more common with consumption of sugar-sweetened alcoholic beverages (Albers *et al.,* 2015; Crews, 2015; Siegel *et al.,* 2016).

2.1.3 Popularity and marketing strategies

Sweetened alcoholic beverages are sold all over the world, with production and marketing strategies differing according to national legal constraints (Mosher & Johnsson, 2005). Studies indicate that underage drinkers find sweetened alcoholic beverages appealing, and therefore, businesses employ marketing strategies that seem to target young people (Mosher & Johnsson, 2005). The placement of the

marketing messages in the media constitutes the promotion component of overall marketing. Producers of sweetened alcoholic beverages, like those that promote conventional alcohol, depend increasingly on non-traditional media, including the Internet, commercial placement in motion pictures, and sponsorship of concerts and athletic events to promote these products and increase their popularity (WHO, 2022). Due to another significant global development in recent decades, the rise of social media and "electronic commerce" on the internet, the area of advertising and promotion now encompasses much more than only state-regulated media (Room & O'Brien, 2021). Thus, newer marketing strategies include social media, text messaging, podcasting, email campaigns, and short message service (SMS) campaigns (WHO, 2022).

One of the main goals for the manufacturers is to have these sweetened alcoholic beverage products easily available, particularly in places where young people are likely to congregate, such as convenience stores, supermarkets, and fuel stations (Mosher & Johnsson, 2005). Another marketing strategy that makes sweetened alcoholic beverages more popular among younger people is the cost. Because of the sensitivity of the youth to cost, sweetened alcoholic beverages are usually less expensive than conventional alcohol. This is because some regulating authorities do not consider them as actual alcoholic beverages and therefore tax them lower than conventional alcohol (Mosher & Johnsson, 2005; Diloreto *et al*., 2012). These factors make sweetened alcoholic beverages more popular and widely consumed than conventional unsweetened alcohol, among younger adults and adolescents.

Although there is a paucity of information about sweetened alcohol consumption statistics in South Africa, the WHO has placed South Africa among the top countries with a higher prevalence of alcohol consumption (WHO, 2017). Approximately 41% of

the South African population is reported to consume alcohol, with 20-30% of them engaged in heavy drinking (Mafa *et al.,* 2019). In South Africa, the consumption of sugar-sweetened beverages increased in both urban and rural areas, contributing to a higher risk of non-communicable diseases. This trend places a serious economic burden on the country and strains the healthcare system (Vorster *et al.,* 2014; Bosire *et al.,* 2020)."

2.1.4 Under-age drinking: a ticking time bomb

There is a growing concern about the implications of flavoured alcoholic beverages replacing soft drinks among teenagers and their potential to exacerbate alcohol-related issues among these young people (Mosher & Johnsson, 2005). The fact that the majority of sweetened alcoholic beverages do not taste like alcohol makes them a dangerous source of alcohol. Alcoholic beverages that do have a noticeable alcohol taste are typically produced by adding flavourants to the alcohol, which also covers up the natural flavour of the alcohol (Crews, 2015). While some types of alcohol may require an "acquired taste", drinking is made simpler when the strong liquor flavour is covered up by a more robust sweet taste, which facilitates the consumption of large amounts of alcohol (Crews, 2015), thus, potentially increasing the development of alcohol-related diseases.

The fact that most sweetened alcoholic beverages don't taste like conventional alcohol, people may underestimate how much alcohol they consume, which could lead to overconsumption and related health problems (Binakonsky *et al*., 2011; Siegel *et al.,* 2016).

Consumption of sweetened alcoholic beverages has caused several alcohol poisoning cases, including some fatalities, caused by binge drinking (Crews, 2015). Based on findings from studies that investigated sweetened alcohol and its relation to alcohol

consumption, it is believed that the consumption of sweetened alcoholic beverages leads to increased alcohol consumption that eventually leads to binge or heavy drinking (Binakonsky *et al.,* 2011). Binge drinking, heavy alcohol consumption, or inappropriate alcohol consumption may be associated with an increased burden of cardiometabolic diseases (Piano, 2017). There are still unanswered crucial concerns, such as how different types of alcohol and drinking habits affect cardiometabolic outcomes (Li *et al*., 2023). Studies have argued that the negative or positive effects of alcohol depend on the type of alcoholic beverages and the amount consumed (Luo *et al*., 2020; Estruch & Hendriks, 2022). However, such findings are limited in their description of how sweetened alcohol affects different cardiometabolic indices.

2.2 Cardiometabolic diseases: overview and risk factors

Cardiometabolic diseases (CMDs) are a group of conditions that increase the risk of cardiovascular diseases (CVDs) and metabolic disorders (Kulkarni & Sarin, 2023). These disorders include coronary artery disease (CAD), heart failure, stroke, obesity, dyslipidaemia, type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD) which is now renamed to metabolic dysfunction associated steatotic liver disease (Kulkarni & Sarin, 2023), chronic kidney disease (CKD) and associated risk factors (Guan *et al*., 2022). The spectrum of CMDs evolves to a clinically recognisable high-risk state of metabolic syndrome and prediabetes, T2DM, and CVDs, with insulin resistance as the starting point in most cases (Guo *et al*., 2014). When compared with other global health concerns, the growth in CMDs, which are responsible for a large share of the non- communicable diseases (NCDs) burden, has been associated with minimal outcry and little funding, and as a result, they have become the leading cause of mortality worldwide (Ralston & Nugent, 2019). CMDs are believed to be the leading cause of death and disabilities globally (Guan *et al.,* 2022). The prevalence of

cardiometabolic disorders is still rising globally despite the remarkable progress made in the management of these conditions (Sattar *et al*., 2020; Flood *et al*., 2022). According to the WHO, cardiometabolic diseases account for roughly 32% of all deaths worldwide (WHO, 2022). As an important component of non-communicable diseases, the development of CMD is associated with different risk factors (WHO, 2022).

More than half of the worldwide burden of NCDs is attributable to the four primary modifiable risk factors for CMDs: unhealthy eating, smoking, excessive alcohol consumption, and physical inactivity (WHO, 2022). These factors have made CMD more widespread and deadlier globally, particularly in low- and middle-income countries(Wang *et al*., 2023). Alcohol consumption and dietary habits have been listed among the major risk factors for CMD (Raffa *et al*., 2017; Stewart *et al*., 2017). An increase in the prevalence of CMDs is connected with greater availability of sugar, especially in the form of sweetened beverages, refocusing public health attention on the relationship between increased sugar consumption and cardiometabolic diseases (Herman & Birnbaum, 2021).

2.2.1 Evidence on the relationship between sweetened alcoholic beverages and cardiometabolic health

Increased consumption of both alcohol (Du *et al*., 2017), and fructose (Bernardes *et al*., 2016), has been associated with increased risk of different indices of cardiometabolic risk factors. A study where rats were fed with sweetened alcohol (fructose + ethanol), showed an increased risk of cardiometabolic risk factors such as dyslipidaemia, insulin resistance and liver damage (Alwahsh *et al.,* 2014) compared to controls. Over decades of epidemiological research, alcohol intake has been linked to several characteristics associated with cardiometabolic health in an inverse or J-shaped relationship (Lankester *et al.,* 2021). These correlations have led to the theory

that moderate drinking is good for cardiometabolic health (Lankester *et al*., 2021). However, it is argued that this interpretation is flawed because the relationship does not match the data for several established risk factors. For instance, alcohol use has been directly linked to outcomes like hypertension, regardless of the amount consumed (Fuchs & Fuchs, 2021). Due to the caloric content of alcohol, excessive consumption can lead to obesity, which can result in obesity-related complications such as insulin resistance and hypertension (Fan *et al*., 2019). In a dose-dependent manner, alcohol consumption has been associated with an increased risk of metabolic syndrome, hypertension (Du *et al.,* 2017), and type 2 diabetes mellitus (Li *et al*., 2016). Chronic alcohol consumption has also been linked to pancreatic β-cell dysfunction, inducing insulin resistance and increasing the risk of type 2 diabetes mellitus (Jang *et al*., 2019).

In the cardiovascular system, regular excessive alcohol consumption is toxic and can aggravate pre-existing cardiac conditions (Marcos *et al*., 2021). Alcohol consumption is physiologically believed to be associated with increased blood clotting and reduced ventricular fibrillation threshold (Rehm *et al*., 2010). According to several epidemiological and animal studies, excessive alcohol consumption may impair cardiac function and raise the likelihood of heart failure and cardiomyopathy (Larsson *et al*., 2014).

The toxicity produced by excessive fructose consumption has been compared to that of increased alcohol consumption. This includes hypertension, a rise in uric acid, elevated triglycerides, increased fat synthesis in the liver, insulin resistance, and damage to macromolecules such as lipids, proteins, and DNA (Lustig *et al*., 2012). Increased fructose consumption has been associated with an increased risk of cardiometabolic dysfunction, as it is related to metabolic syndrome, oxidative stress, inflammation, and hypertension (Bernardes *et al.,* 2016), and insulin resistance (Li *et al*.,

2016; Kovačević *et al*., 2021). All of which increases the risk of cardiovascular diseases (Bernardes *et al.,* 2016). Based on epidemiological studies from large cohorts, there is a correlation between fructose (Jalal *et al*., 2010) or sugar (Te Morenga *et al*., 2014) consumption and hypertension. A study that explored the relationship between the consumption of sweetened beverages and the risk of coronary heart disease (CHD), reported a positive link between the consumption of sugar-sweetened beverages and increased risk of CHD by facilitating increased blood lipids, inflammatory markers, and leptin (De Koning *et al*., 2012).

2.2.2 Findings from human and animal studies related to sweetened alcohol and cardiometabolic diseases

2.2.2.1 Sweetened alcohol and liver diseases

Although a lot of information has been published regarding the effects of alcohol and fructose separately on hepatocellular metabolism, the long-term effects of both substances remain poorly understood (Alwahsh *et al.,* 2014). A study reported that both fructose and ethanol undergo a comparable hepatic metabolism in that they both cause lipogenesis and fructosylation of proteins, which produce superoxide in the same manner as acetaldehyde, a metabolite of ethanol (Rouault, 2003). Feeding rats with ethanol combined with fructose resulted in some degree of hepatic inflammation and portal fibrosis (Alwahsh *et al.,* 2014). Additionally, the combination of alcohol and fructose significantly raised the expression of metabolic and inflammatory regulators (such as lipocalin-2 and melanocortin 4 receptors) in the liver (Alwahsh *et al*., 2014; Malik & Hu, 2012).

Research has indicated that a high-fructose diet may be harmful to the liver (Muriel *et al.,* 2021). When excessive amounts of fructose enter the liver, it is converted into fat via lipogenesis (Jang *et al*., 2020). Consumption of excess fructose may eventually

lead to the development of non-alcoholic fatty liver disease (NAFLD), a condition where the liver cells retain an excessive amount of fat (Jang *et al*., 2020). Elevation of uric acid in the liver could also potentially boost lipogenic enzymes and fructose metabolism, further augmenting *de novo* lipogenesis, and this could explain how it contributes to non- alcoholic fatty liver disease (Wei *et al*., 2020). Fructose influences a multitude of signalling pathways that are proinflammatory, fibrogenic, and carcinogenic, which explains why it has negative effects on the body, particularly the liver (Muriel *et al.,* 2021). Fructose was also reported to impair liver regeneration in Sprague-Dawley rats fed with a high fructose diet compared to the control (Tanoue *et al*., 2011).

Although the liver is thought to be the only totally regenerating organ in the human body, chronic liver disorders like alcoholic liver disease (ALD) impede hepatocyte regeneration(Wu *et al*., 2023). Alcohol consumption is one of the major causes of liver disease and liver damage. The International Agency for Research on Cancer (IARC) has identified alcohol as a human liver carcinogen (Lam *et al*., 2021). Due to its hepatotoxicity, alcohol is one of the main factors in liver-related deaths across the globe (Molina *et al*., 2023).

Both ethanol and fructose are primarily metabolised in the liver and are recognised to play a role in the onset of various stages of liver disorders. They can cause inflammation in the liver and raise the release of proinflammatory mediators, which in turn encourage hepatocyte damage and death and ultimately lead to more advanced liver diseases (Shulga & Pastorino, 2014). Combining them (alcohol and fructose) in the form of sweetened alcohol may potentially increase the toxic and metabolic burden on the hepatocytes. Another metabolic target of alcohol and sugar is adipose tissue associated with adiposity.

2.2.2.2 Sweetened alcohol and obesity

Obesity refers to the excess accumulation of body fat to the detriment of the general health and well-being of the individual, and it usually happens when the total energy intake exceeds the total energy expenditure (Niklas *et al*., 2020). Obesity results from a multitude of physiological, environmental, behavioural, and sociopolitical factors, all of which result in a positive energy balance (Malik & Hu, 2022).

Obesity is a risk factor for insulin resistance and metabolic syndrome (Guo *et al.,* 2014), hypertension, increased blood cholesterol, decreased high-density lipoprotein cholesterol (HDL-c), and increased blood glucose concentration (Carey *et al*., 1997), which contribute to cardiometabolic diseases.

There is a dearth of literature on the effects of sweetened alcohol on obesity. However, considered individually, sugar, particularly orally consumed fructose, stimulates the autonomic and endocrine responses resulting in the downregulation of the cephalic phase of the insulin pathway in taste cells, hence lowering the generation of pancreatic insulin (Glendinning *et al*., 2017). Furthermore, consuming fructose raises ghrelin levels in the serum and decreases leptin and glucagon-like peptide 1 levels, which makes eating fructose more stimulating than eating glucose (Bodnaruc *et al*., 2016). Ghrelin increases the neuronal activity of neuropeptide Y, which increases appetite, while suppression of glucagon-like peptide 1 results in a decrease in insulin secretion (Koliaki *et al*., 2010). All these mechanisms are associated with positive energy balance, which is associated with obesity.

There is a strong relationship between heavy alcohol consumption (consumption of more than 3 drinks per day) (Abel, 1998), and obesity as it relates to anthropometric determinants of obesity such as body mass index, waist circumference, and hip-waist circumference (Coulson *et al*., 2013; Traversy & Chaput, 2015). Most of the studies

conducted in humans are in support of the fact that light {(consumption of less than 2 drinks per day) (Abel, 1998)} to moderate alcohol consumption {(consumption of more than 3 drinks per day) (Abel, 1998)} does not relate to increased weight gain (Traversy & Chaput, 2015; Yeomans, 2010), as compared to heavy consumption which has a strong relationship with obesity and weight gain (Golzarand *et al*., 2022). This can be related to increased alcohol consumption, as it is a known fact that 1g of alcohol provides 7.1 kcal of energy, which is almost double the energy provided by 1g of carbohydrate or protein (Hendriks, 2020).

The mechanism through which sweetened alcohol regulates appetite may be related to appetite-regulating hormones. While leptin suppresses appetite, it has been reported that fructose-fed rats showed raised leptin levels compared to controls (Alwahsh *et al.,* 2014), while ghrelin, a hormone that stimulates appetite, has been shown to decrease following alcohol consumption (Yeomans, 2010). Therefore, through alcohol and fructose synergistic effects, sweetened alcohol (alcohol + fructose) may have decreased appetite, leading to decreased food intake.

The most prevalent metabolic condition associated with obesity is insulin resistance/hyperinsulinemia, which is the primary factor contributing to the development of another component of CMDs – dyslipidemia (Vekic *et al*., 2019).

2.2.2.3 Sweetened alcohol and dyslipidaemia

Dyslipidaemia is an increased plasma level of triglycerides (TG), decreased high-density lipoprotein-cholesterol (HDL-c), and increased low-density lipoprotein (LDL) (Teramoto *et al*., 2012). It is regarded as a significant modifiable risk factor for cardiovascular diseases (Addisu *et al*., 2023). Dyslipidaemia causes cholesterol and other fats to accumulate in the arterial walls, resulting in plaques that constrict the arteries and limit blood flow. Plaque formation can result in numerous cardiovascular

conditions, such as peripheral artery disease, coronary artery disease (which results in a heart attack), and stroke (Du & Qin, 2023).

Five different diets were used in a particular study. These diets include chow (control), liquid Lieber-DeCarli (LDC) diet, LDC +30%J of ethanol or fructose, and LDC combined with 30%J ethanol and 30%J fructose. In this study, dyslipidaemia was reported in rats fed with sweetened alcohol (a combination of alcohol and fructose), and this was ascribed to fructose and alcohol's synergistic relationship (Alwahsh *et al.,* 2014). Fructose co-administered with alcohol has also been reported to be associated with increased serum triglycerol concentration, contributing to dyslipidaemia and metabolic syndrome (Uzuegbu & Onyesom, 2009). Both fructose and alcohol facilitate *de novo* lipogenesis, causing increased free fatty acid formation that worsens dyslipidaemia (Alwahsh *et al.,* 2014).

De novo lipogenesis (DNL), which turns fructose carbons into fatty acids, is projected to be the cause of elevated hepatic triglyceride production and VLDL-triglyceride secretion, which in turn facilitates the appearance of circulating triglycerides (Taghibiglou *et al*., 2000). In a human study that compared the effect of consumption of glucose- and fructose-sweetened beverages, it was reported that hepatic *de novo* lipogenesis, postprandial triglycerides, and indicators of lipid metabolism, including small LDL particles and fasting apoB, were markedly increased in the fructose-consuming group (Stanhope *et al*., 2009). Thus, associating fructose with the development of dyslipidaemia.

While moderate alcohol use is known to raise HDL cholesterol levels, excessive alcohol drinking raises body weight and triglyceride levels (Klop *et al*., 2013). The risk of dyslipidaemia increases with heavy alcohol consumption due to a reduction in HDL-c (Capurso & Petrakis, 2016). Several studies have demonstrated that long-term

alcohol consumption activates the hepatic proprotein convertase subtilisin/kexin type 9 (PCSK9) enzyme, which suppresses the extracellular signal-regulated kinase (ERK) activation that is linked to decreased LDL receptor expression in the liver and then downregulates the LDL-receptor (Wang *et al.,* 2010). Alcohol causes inhibition of the activity of lipoprotein lipase, which then causes decreased clearance of triglycerides and induces hypertriglyceridemia (Zemánková *et al*., 2015).

In a patient with a history of heavy alcohol consumption who demonstrated reversed hypertriglyceridaemia following alcohol withdrawal without any treatment, laboratory parameters showed that alcohol increased the level of cholesterol, decreased HDL-c, and caused hypertriglyceridaemia (Capurso & Petrakis, 2016). In an animal study using male and female Wistar rats, chronic alcohol consumption significantly altered the levels of different types of lipids, such as diacylglycerol, lysophosphatidylcholine, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol (Li *et al*., 2018). Low HDL levels, elevated levels of small dense LDL particles, and elevated triglyceride levels are important characteristics related to diabetes mellitus (Bahiru *et al*., 2021).

2.2.2.4 Sweetened alcohol and diabetes mellitus/insulin resistance

Diabetes mellitus occurs as a result of impaired insulin secretion and/or resistance (Tatsumi *et al*., 2018). Increased fructose consumption has been linked to several metabolic problems, such as type 2 diabetes mellitus (T2DM) and insulin resistance (Elliott *et al*., 2002). Fructose consumption can result in an ‘energy deficit’ or an impaired ATP synthesis (Abdelmalek *et al*., 2012) as a result of rapid phosphorylation of fructose. Consequently, decreased hepatic ATP can result in reduced utilisation of insulin, which may potentially result in fewer insulin receptors, causing insulin resistance (Helsley *et al.,* 2020). Additionally, fructose decreases hepatic fatty acid

oxidation and increases hepatic *de novo* lipogenesis, both of which might result in a greater buildup of fat in the liver, which in turn causes inflammation and hepatic insulin resistance (Muriel *et al.,* 2021). Increasing hepatic insulin resistance stimulates pancreatic β-cells to secrete more insulin, which can lead to β-cell dysfunction that worsens over time (Buchanan *et al*., 2002). Deterioration in β-cell activity over time might result in insufficient insulin production, exacerbating oxidative stress and inflammation brought on by fructose and worsening hepatic insulin resistance (Hummasti & Hotamisligil, 2010). Overall, the effects of consuming too much additional fructose include disruption of the body's hepatic and systemic metabolism as well as widespread insulin resistance (DiNicolantonio *et al*., 2015). An overwhelming body of research indicates that consuming more sugar, especially fructose, increases the risk of developing T2DM and weight gain (Helsley *et al*., 2020).

A systematic review and dose-response meta-analysis on the association between alcohol consumption and the risk of T2DM reported that light to moderate alcohol consumption is related to a lower risk of T2DM (Li *et al.,* 2016). This may be because drinking alcohol in moderation has been linked to improved insulin sensitivity and decreased fasting insulin resistance, both of which may have significant effects on the development of T2DM, and show no sexual dimorphism (Li *et al.,* 2016). In a Mendelian randomisation analysis on alcohol consumption and risk of diabetes in a Chinese population, an increased risk of diabetes and insulin resistance was observed with increased alcohol consumption, with the result more significant among men than women, showing aggravation of other diabetic-associated traits even among moderate drinkers (Peng *et al*., 2019). In a prospective study that investigated the relationship between alcohol and impaired insulin secretion and insulin resistance in a Japanese population, it was reported that moderate and heavy alcohol consumption was associated with impaired insulin secretion and insulin resistance, thereby increasing

the risk of development of T2DM (Tatsumi *et al*., 2018). Another study also showed that light to moderate consumption of alcohol reduces the risk while heavy alcohol consumption increases the risk of diabetes (Polsky & Akturk, 2017).

When considered collectively, it has been shown that alcohol and fructose combined as sweetened alcohol can, through synergistic interaction, cause hepatic insulin resistance, diabetes, and liver disease (Alwahsh *et al.,* 2014). Also, the co-administration of alcohol and fructose has been shown to cause impaired glucose levels and increased TG concentrations, an effect that increases the risk of diabetes mellitus (Uzuegbu & Onyesom, 2009). Sweetened alcohol, therefore, may have the potential to cause an increased risk of diabetes mellitus. However, some sweeteners used to prepare sweetened alcohol, like polyols/sugar alcohol (example: lactitol, mannitol, xylitol, maltitol, isomalt, erythritol, and sorbitol), are considered sugar-free with a low glycaemic index and have been shown to be safe in diabetic patients (Msomi *et al.,* 2021). However, studies have also shown these sweeteners to have some impact on insulinemia and postprandial glycaemia, (Msomi *et al.,* 2021), and therefore, excess consumption is discouraged.

Gender difference is also reported in relation to alcohol metabolism and diabetes mellitus. Sex hormones significantly impact energy metabolism, body composition, vascular function, and inflammatory responses (Ciarambino *et al.,* 2022). Alcohol dehydrogenase activity in the stomach is lower in women than in men, which raises the bioavailability of ethanol in women. Thus, for similar consumption, women's blood alcohol levels are greater due to a 7.3% reduction in the volume of ethanol distribution compared to men (Gimunová *et al.,* 2022). Additionally, gastric emptying time is slower in females, resulting in higher blood alcohol concentration in females compared to males (Gimunová *et al.,* 2022). However, in relation to diabetes, more males have diabetes before puberty, whereas more females have it after menopause and in old

age, indicating women have some protection against diabetes due to sex hormones (Ciarambino *et al*., 2022).

Cumulatively, it has been shown that fatty liver, hyperglycaemia, dyslipidaemia, and hyperinsulinemia are all linked to insulin resistance and metabolic syndrome. The cumulative effect of these factors raises the risk of cardiovascular disease (Shimomura & Wakabayashi, 2013).

2.2.2.5 Sweetened alcohol and cardiovascular diseases

Fructose (Mirtschink *et al*., 2018) and alcohol (Piano *et al*., 2018) consumption has been implicated in different cardiovascular diseases such as hypertension, vascular dysfunction, myocardial infarction and arrhythmias. The various cardiovascular diseases related to alcohol and fructose consumption resulted from alterations in the structural and functional status of the heart, which ultimately resulted in heart failure (Piano *et al*., 2018; Groenewegen *et al*., 2020; Busnatu *et al*., 2022). There is an increasing amount of evidence to suggest that both alcohol (Larsson *et al*., 2014) and fructose (Zhang *et al*., 2024) contribute to heart failure.

Given the growing number of individuals with heart failure, heart failure continues to be a major clinical and public health problem (Groenewegen *et al*., 2020). Heart failure affects 1-12% of the adult population, depending on the type, region, sex, and ethnicity (Roger, 2021), and it is also showing an increasing trend in South Africa (van Rensburg, 2022).

According to the European Society of Cardiology, heart failure is a clinical disease that results from structural and functional abnormalities of the heart, causing decreased contractile ability of the heart and increasing intracardiac pressures (Ponikowski *et al*., 2016). This may alter the amount of blood pumped by the ventricles, called ejection

fraction (EF). Usually, this is a condition that results in systolic and/or diastolic ventricular dysfunction (Schwinger, 2021).

Heart failure may be present in two forms based on the measurement of LV ejection fraction (LVEF) (Ponikowski *et al.,* 2016). Heart failure with preserved ejection fraction (HFpEF), is seen in individuals with normal ejection fraction (≥50%), and heart failure with reduced ejection fraction (HFrEF) is seen in individuals with reduced ejection fraction (<40%). HFpEF presents normal LV cavity size but with an increased LV wall thickness, resulting in LV diastolic dysfunction and an increase in filling pressure. On the other hand, HFrEF is associated with increasing LV dilatation and detrimental cardiac remodelling (Murphy *et al*., 2020), and systolic dysfunction (Ponikowski *et al*., 2016).

Different studies have reported various findings linking alcohol and fructose consumption to the development of systolic and diastolic dysfunction. It has been demonstrated that drinking alcohol results in severe cardiac steatosis, mitochondrial dysfunction, contractile dysfunction, impaired myocardial relaxation, increased arterial elastance, and a decrease in total peripheral resistance (Matyas *et al*., 2016). Alcohol induces vascular responsiveness that affects the function of coronary arteries and the heart, resulting in heart failure (Piano 2018). An increase in alcohol consumption has been related to increased LV hypertrophy, an increase in relative wall thickness, and causes a decrease in LV diastolic function (Park *et al.,* 2018). Regardless of age, body mass index, blood pressure, insulin sensitivity, or left ventricular mass index, alcohol consumption was associated with diastolic dysfunction(Catena *et al*., 2016).

In a population-based study, LV systolic function has also been related to increased alcohol consumption (Yousaf *et al*., 2014).

As seen with alcohol, the increase in fructose consumption has also been related to structural and functional abnormalities of the heart. Fructose consumption is associated with apoptosis, inflammation, lipid buildup, metabolic disruption, and pathological growth in the heart (Annandale *et al*., 2021). A high-fructose diet not only caused inflammation but also heart failure (Wang *et al.,* 2023). Recent research revealed that cardiomyopathy brought on by high fructose consumption contributes to systolic and diastolic dysfunctions (Wang *et al*., 2023). Fructose consumption has been related to increased LV hypertrophy (Wu *et al.,* 2018), increasing the risk of diastolic dysfunction. Fructose also decreases the fatty acid oxidation flux rate, which interferes with LV function, leading to heart failure (Zhang *et al.,* 2024). Increased fructose consumption has been shown to exacerbate LV eccentric hypertrophy and reduce LV function (Bouchard-Thomassin *et al*., 2011). Fructose consumption has also been associated with increased cardiac fibrosis and worsened diastolic dysfunction (Xing *et al*., 2010).

Increased alcohol consumption has been associated with increased risk of clinical and subclinical left ventricular dysfunction (Piano, 2002). Alcohol causes direct injury to the myocardium by stimulating the renin-angiotensin system and causes oxidative stress in the heart (Domínguez *et al.,* 2024). Acetaldehyde, a byproduct of alcohol metabolism, can cause mitochondrial dysfunction and cardiac injury by impairing actin-myosin interaction, leading to heart failure (Domínguez *et al.,* 2024). As a proinflammatory agent, acetaldehyde has been associated with heart failure with preserved ejection fraction (HFpEF) (Wong *et al.,* 2024).

Overconsumption of fructose causes oxidative stress, changes energy metabolism, and causes cardiac hypertrophy, all of which lead to heart failure (Zhang *et al.,* 2024). Increased fructose consumption causes detrimental energy shifts in an overworked heart, which could result in the accumulation of dangerous lipids and unchecked

cardiac hypertrophy that could eventually cause heart failure (Daniels *et al.,* 2021). By causing cardiac inflammation, changing heart metabolism, and fostering conditions like obesity and hypertension that are risk factors for heart failure with reduced ejection fraction (HFrEF), excessive fructose consumption may play a role in the onset and exacerbation of HFrEF. According to research conducted on both human patients and animal models, high fructose diets are associated with decreased cardiac function, elevated heart inflammation, and inhibited fatty acid oxidation (Zhang *et al.,* 2024).

Other cardiovascular risk factors (such as hypertension, dyslipidaemias, diabetes, and obesity) have been associated negatively with excess alcohol and fructose consumption, which can worsen heart failure. Fructose and alcohol have been shown to induce insulin resistance, diabetes, and liver disease (Alwahsh *et al.,* 2014). Co-administration of alcohol and fructose has been shown to cause impaired glucose levels and increased TG concentrations, an effect that increases the risk of diabetes mellitus (Uzuegbu & Onyesom, 2009). In an animal model, after 10 days of intervention, it was demonstrated that a diet containing 5% alcohol and one episode of binge drinking at a rate of 5 g/kg body weight resulted in significant mitochondrial dysfunction, cardiac steatosis, contractile impairments, as well as impaired relaxation and arterial elastance and a decrease in total peripheral resistance, which can lead to heart failure (Matyas *et al.,* 2016). Chronic alcohol exposure in other mouse models resulted in cardiac fibrosis (Liu *et al.,* 2011), worsened the progression of fibrosis and heart failure following myocardial infarction (Liang *et al.,* 2020), and raised blood pressure, causing alcohol-induced hypertension, which can worsen heart failure (Andersson *et al*., 2022).

Considering the individual effects of fructose and alcohol on cardiac function, the combined effects of fructose and alcohol need to be further investigated on the heart

as there is currently limited evidence to explain fully the combined effects in the form of sweetened alcohol.

2.2.2.6 Sweetened alcohol, gut microbiota, and cardiometabolic diseases

The gastrointestinal tract (GIT) microbiota is made up of a large variety of species of bacteria, fungi, archaea, and protozoa that coexist in a community and interact with the host organism (Matijašić *et al*., 2020). Healthy gut microbiota promotes the production of immune cells, maintains the intestinal mucosal barrier, and aids in the digestion of foods and medications (Jandhyala *et al*., 2015). Before birth, commensal bacteria begin to colonise the GIT, and both in newborns and adults, the gut microbiota and its byproducts increase the number of immune cells (Luo *et al*., 2015). Immune cells' interactions with the microbiome may influence how CVD develops (Kazemian *et al*., 2020). Endotoxins or metabolites from the gut microbiota can enter the bloodstream and cause metabolic inflammation, particularly in people with a risk of CVD, like obesity (Chen *et al*., 2018). The pathogenesis of CMDs is thought to be influenced by irregularities/alterations in the gut microbiota range and microbial metabolism (Tang *et al*., 2019).

According to recent studies, fructose consumption modifies the gut microbiota and the bacterial metabolites that they produce, which encourages the onset and spread of non-alcoholic steatohepatitis (NASH) (Lambertz *et al*., 2017). Consuming too much fructose reduces the expression of intestinal tight junction proteins, including E- E-cadherin, β-catenin, occludin, zonula occludens 1, and junctional adhesion molecule A (Rahman *et al*., 2016; Cho *et al*., 2021). This situation causes dysbiosis by increasing the number of '*Bacteroides*, *Proteobacteria*, *Enterobacteria, Escherichia*, *Blatitia producta*, and *Bacteroides fragilis* and decreasing the number of *Actinobacteria*, *Akkermansia*, *Verrucomicrobia*, *Coprococcus eutactus,* and *Lactobacillus' (Do et al.,*

2018; Mirtschink et al., 2018). This increases the loss and blebbing of the laminar propria, which causes inflammation in the small intestine, and increases the possibility that toxic bacterial metabolites will reach the liver, further exacerbating inflammation in NASH (Do *et al.,* 2018; Mirtschink *et al.,* 2018).

Following alcohol intake, the small intestine and stomach are the primary locations from where ethanol is taken into the bloodstream. A growing body of research indicates that ethanol is directly harmful to the GIT before it enters the liver for metabolism (Silva *et al*., 2021). Gut microbiota is also impacted by various drinking behaviours and amounts of ethanol consumed over time (Giuffrè *et al*., 2020). Alcohol specifically destroys the intestinal protective mucus layer, reduces gastric acid output, promotes the growth of microorganisms, particularly gram-negative bacteria, increases intestinal permeability and impairs the immune system (Giuffrè *et al*., 2020). Thus, chronic ethanol consumption can alter bacterial populations and host gene expression, thus disrupting protein transport (for example, gap junction protein GJA5) and metabolism (Barr *et al*., 2018). Alcohol-induced changes in the composition of the gut microbiota can lead to cardiometabolic disorders such as atherosclerosis, hypertension, and heart failure (Silva *et al.,* 2021). Through these mechanisms, alcohol consumption can alter the gut microbiota system and can lead to an increased risk of cardiometabolic diseases (Attaye *et al*., 2020).

Therefore, looking at the negative individual effects of alcohol and fructose on gut microbiota and cardiometabolic diseases, combining them in the form of sweetened alcohol will most likely increase the risk of cardiometabolic diseases by facilitating the growth of unfavourable microbiota, which leads to a leaky gut and entry of harmful substances such as acetate into the liver, which has negative inflammatory effects, oxidative stress, and ROS formation.

2.3 Potential mechanisms underlying the effects of sweetened alcoholic beverages on cardiometabolic health

2.3.1 Overview of fructose and alcohol metabolism

Both sugar and alcohol facilitate the progression of cardiometabolic diseases by increasing the formation of ROS, which increases the production of inflammatory mediators, mitochondrial dysfunction, ATP depletion, and apoptosis, which are the major drivers of the increased risk of cardiometabolic diseases (Ojeda *et al*., 2022). There is mounting data that suggests that the fructose component of sucrose, in particular, plays a significant role in the pathophysiologic effects of high sugar intake (Alberti *et al.,* 2009). The cardiometabolic effects caused by fructose are directly related to its metabolism. Unrestricted hepatic fructose uptake and metabolism, which is mediated by glucose transporter-2 (GLUT2) and ketohexokinase-C (KHK-C), from increased fructose intake, results in intrahepatic lipid buildup, dyslipidaemia, reduced insulin sensitivity, and hyperuricemia (Lelis *et al*., 2020; Herman & Birnbaum, 2021). Hypoxia-inducible factor subunit alpha (HIF1-α)-driven activation of fructose uptake and metabolism in the heart promotes anabolic cardiac development as well as systolic and diastolic dysfunction in response to cardiac stress from excess fructose consumption (Herman & Birnbaum, 2021). From these mechanisms, high fructose consumption causes vascular dysfunction, insulin resistance, metabolic syndrome, hypertension, inflammation, and cardiac hypertrophy, ultimately resulting in cardiac fibrosis and hypertrophy (Cheng *et al.,* 2021). Furthermore, because fructose is involved in the inflammatory response, excessive fructose consumption speeds up inflammation in the gut, liver, kidney, heart, and brain (Zhang *et al*., 2017) Cheng *et al*., 2021).

Fructose goes through an intermediate three-step metabolism in the liver, producing triosephosphates that enter the route of glycolysis. Fructokinase, the primary phosphorylating enzyme that converts fructose to fructose-1-phosphate, is uncontrolled; hence, it has been suggested that consuming fructose may decrease hepatic ATP pools, which will increase hepatic nucleotide turnover and uric acid generation (Sun & Empie, 2012). Due to its powerful activation of the Node-Like Receptor family pyrin domain containing 3 (NALP3) inflammasome, increased uric acid may cause a low-grade chronic inflammation. This low-grade inflammation is primarily responsible for developing insulin resistance (Lanaspa *et al*., 2012) which typically serves as the initial stage that leads to different cardiometabolic diseases (Guo *et al*., 2014).

After its consumption, alcohol is metabolised by alcohol dehydrogenase to acetaldehyde, a highly toxic chemical and proven carcinogen, which then undergoes additional metabolism by the action of aldehyde dehydrogenase to produce acetate, a different byproduct that is less active (Molina *et al.,* 2018; Wu *et al*., 2023). This facilitates the reduction of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dehydrogenase (NADH) (Jiang *et al.,* 2020), which leads to the formation of ROS, which reduces the formation of endothelial nitric oxide synthase (eNOS) (Chao *et al.,* 2010). The reduction in the formation of eNOS then negatively affects different organs in the body, with the liver being primarily affected, as greater than 90% of alcohol is metabolised in the liver (Ojeda *et al.,* 2022). While nitric oxide plays an important role in liver function, increased ROS formation interferes with nitric oxide activity, leading to oxidative stress. An increased oxidative stress can cause proinflammatory mediators, which can damage the hepatocytes (Diesen & Kuo, 2010). Excess consumed alcohol is metabolised in cytochrome P450 2E1 (CYP2E1) in the

endoplasmic reticulum because alcohol dehydrogenase becomes saturated in such situations (Tanaka *et al*., 2000; Wu *et al*., 2023). In the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Teschke, 2019), CYP2E1 causes the generation of more ROS, leading to more injuries to the liver and other vital organs, such as the heart and kidneys (Tanaka *et al*., 2000; Wu *et al.,* 2023).

In excess amounts, alcohol also causes mitochondrial dysfunction by damaging the mitochondrial membrane. This causes ROS formation, reduces ATP production, and induces apoptosis (Kowaltowski & Vercesi, 1999; Zhang *et al*., 2015). Excess alcohol consumption also activates the sympathetic nervous system, the renin-angiotensin-aldosterone system, and the hypothalamic-hypophyseal axis that facilitates an increase in the amount of aldosterone and angiotensin-II released, leading to vasoconstriction in the kidneys and causing increased systolic blood pressure (Jian *et al.,* 2017; Piano *et al.,* 2018). This increased systolic blood pressure is usually the starting point for hypertensive nephropathy. In the same process, the cardiovascular system is adversely affected, thus, increasing the risk for different cardiovascular conditions, such as hypertension (Molina *et al*., 2018), vascular dysfunction (Vaschillo *et al*., 2018), and arrhythmias (Piano *et al*., 2018; Wang *et al*., 2022).

Catalase is the major enzyme that metabolizes alcohol in the heart, and in cases of excess amounts of alcohol being present, CYP2E1 also participates in the metabolism of alcohol, causing more ROS formation (Zhang *et al*., 2013). The generated acetate from the metabolism of alcohol combined with acetate supplied from the blood (Wilson & Matschinsky, 2020), increases ROS production in the mitochondria, leading to mitochondrial oxidation (Ojeda *et al*., 2022). Additionally, excess alcohol consumption leads to activation of Caspase-3 and nuclear factor kappa beta- p65 (NFκB P65). This further maintains ROS production within the heart and leads to the activation of NFκB,

which can activate the release of the injurious cytokines release (Hoffmann *et al*., 2007), which can affect the functions of the heart and lead to different disease conditions.

Three metabolic pathways have been described that involve alcohol and alcohol-related diseases. Alcohol dehydrogenase (ADH), catalase, and the microsomal ethanol oxidation system (MEOS) are the enzymes involved in these pathways (Jiang *et al*., 2020). Free radicals produced by any of these mechanisms have the potential to compromise the antioxidant system and lead to disease conditions (Wilson & Matschinsky, 2020).

The liver converts a large portion of the acetaldehyde to acetate, a significant portion of which exits the liver and travels to peripheral tissues, where it is converted to the essential metabolite acetyl coenzyme A (Wilson & Matschinsky, 2020). The primary metabolite generated by the three main nutrients, fat, carbohydrate, and extra protein, is acetyl CoA (Molina *et al.,* 2023). Thus, alcohol's carbon atoms turn into the same byproducts that are created when protein, fat, and carbohydrates are oxidized, including CO_2, fatty acids, ketone bodies, and cholesterol (Cederbaum, 2012).

Microsomal ethanol oxidation system (MEOS), which involves mainly the cytochrome P450 2E1 (CYP2E1), compromises the defensive systems, which exacerbates oxidative stress both directly and indirectly (Zima *et al*., 2001). CYP2E1 is an important contributor to oxidative stress in the hepatocytes by generating several ROS such as hydroxyl radical (OH−), superoxide (O2 −), hydrogen peroxide (H2O2), and hydroxyethyl radical (HER) (Jiang *et al*., 2020). Figure 2.1 below summarises the mechanisms through which alcohol and excess alcohol consumption interfere with liver function leading to liver damage/disease.

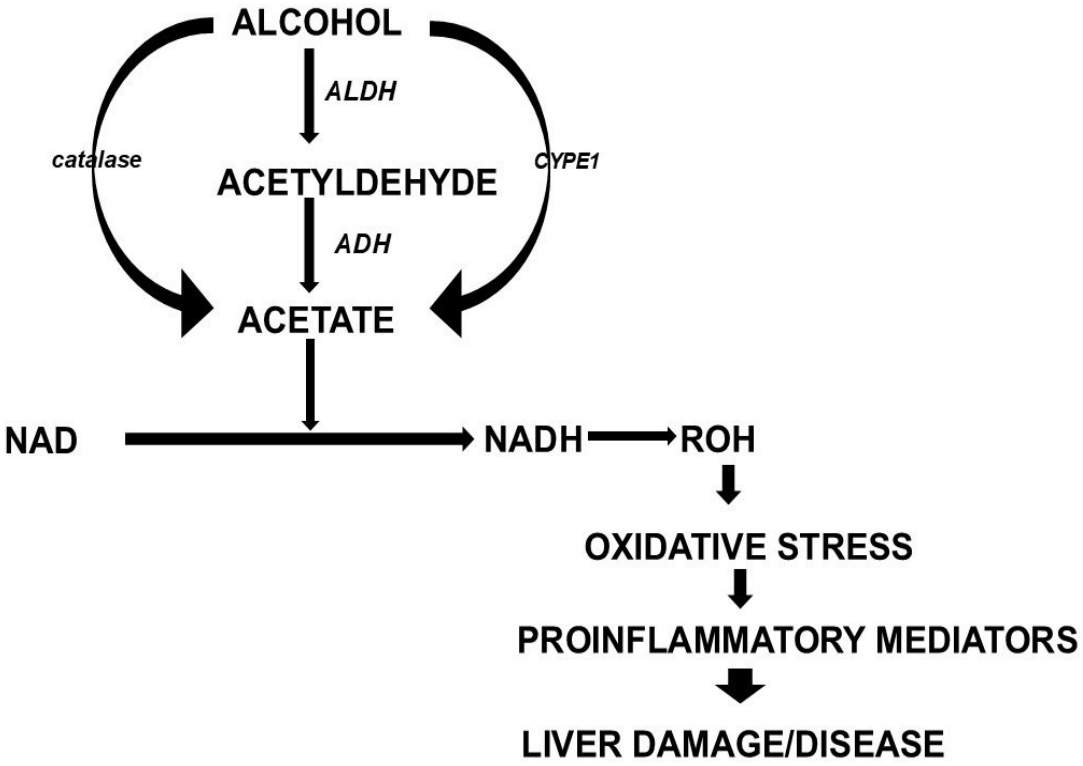

Figure 2.1: Summary of metabolic pathways/process of alcohol and excess alcohol consumption and liver damage. **ADH**: Alcohol dehydrogenase; **ALDH**: Aldehyde dehydrogenase; **CYPE1**: cytochrome P450 2E1; NAD**:** Nicotinamide adenine dinucleotide; **NADH**: Nicotinamide adenine dehydrogenase.

Overall, these suggest that long-term combined use of ethanol and fructose may increase ROS generation, mitochondrial dysfunction, and production of pro-inflammatory mediators leading to increased risk for the development of different cardiometabolic disease variables.

2.3.2 Evidence for potential synergistic or antagonistic effects of fructose and alcohol on cardiometabolic outcomes

Fructose undergoes a ‘Maillard reaction’, which promotes lipid peroxidation and mitochondrial damage and decreases the mitochondrial-biogenic proteins, e.g., peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1α) in the

liver (Alwahsh *et al*., 2014; Rao *et al*., 2020). Consuming ethanol has also been shown to lead to a decrease in hepatic PGC1α, which in turn leads the liver to produce reactive oxygen species (ROS) and iron overload (Zhu *et al*., 2014). According to another study, ethanol may cause hepatic mitochondrial dysfunction, which may affect the mitochondrial DNA by generating protein adducts that hinder the synthesis of ATP and result in a CYP2E1 deficiency (Zhong *et al*., 2014). The breakdown of fructose and alcohol when given simultaneously produces compounds (increase triglyceride, increase blood glucose, increase total cholesterol), that cause oxidative stress and mitochondrial dysfunction (Alwahsh *et al.,* 2014). These results suggest that long-term combined use of ethanol and fructose may increase ROS generation and mitochondrial dysfunction (Alwahsh *et al.,* 2014), which are important mechanisms in the pathophysiology of different cardiometabolic diseases.

Numerous cardiometabolic risk factors, which are all characteristics of CMD, such as insulin resistance and glucose intolerance, hypertension, dyslipidaemia, endothelial dysfunction, obesity, inflammation, and adipocyte dysfunction are significantly influenced by the type of diet and lifestyle of an individual (Alberti *et al.,* 2009). An animal study where rats were fed a combination of fructose and alcohol reported an increased incidence of cardiometabolic risk factors such as insulin resistance, hyperglycaemia, dyslipidaemia, and total cholesterol in the rats (Alwahsh *et al.,* 2014).

2.4 Strategies for mitigating cardiometabolic risk associated with sweetened alcoholic beverage consumption

The fact that people mix their beverages to make cocktails and jello shots at their convenience in addition to commercially available sweetened alcoholic beverages, indicates that little information is available to people with regards to the health consequences of consuming such beverages, which makes them more dangerous

than the conventional alcoholic beverages. As reported in the previous section, fructose and alcohol interfere with different enzymes and metabolic pathways that ultimately affects different body functions and increasing the risk of different cardiometabolic risk factors.

The most popular method used to address different cardiometabolic risk factors is pharmacotherapy (Repas, 2007). However, since a lot of cardiometabolic risk factors co-occur, this strategy might result in polypharmacy, poor patient adherence, and underwhelming results (Grundy, 2006).

A good plan will often involve identifying at-risk individuals, encouraging behaviour modification, and using medicines where necessary. Lifestyle changes are always appropriate in mitigating cardiometabolic diseases. Excessive alcohol consumption is the one modifiable lifestyle factor that is known to contribute to tissue damage and several diseases, including cardiometabolic diseases (Wu *et al.,* 2023). Of importance in lifestyle modification in the management and prevention of cardiometabolic diseases is the control of the increased consumption of sugar/fructose and alcohol as important risk factors for CMD. As practically all physiological systems in the body are influenced by nutrition and because everyone needs to eat and drink every day, dietary interventions are currently the most fundamental and practical lifestyle therapy for promoting cardiometabolic health and avoiding CMDs (Wang *et al.,* 2023).

Regulations for alcohol-sweetened beverages, such as limits on marketing and packaging and the percentage of sugar and alcohol content need to be evaluated and taken into consideration by public health professionals and lawmakers. Governments can mitigate the negative effects of these beverages on health, safety, and socioeconomic issues by developing and enforcing suitable legislation. Policymakers should be urged to implement policies that have proven to be economical and

successful, such as regulation and controlling the promotion of sweetened alcoholic drinks, especially to younger individuals, lowering demand through price and taxation, and expanding knowledge of the negative effects of consumption of these beverages on people's health and social issues that affect both individuals and society.

Studies and information are available regarding the negative individual effects of consuming excess sugar and alcohol. However, with increasing popularity and consumption of sweetened alcoholic beverages, there is a paucity of information on the combined effects of sugar and alcohol in the form of sweetened alcohol on public health. This might hinder implementation of strategies to mitigate the potential negative consequences that might be impacted by sweetened alcohol consumption. Thus, there is need for research to provide evidence-based interventions.

2.4.1 Management of Cardiometabolic Diseases

2.4.1.1 Pharmacological and non-pharmacological management

The management of CMD and its related complications can be pharmacological and/or non-pharmacological, and in some cases, surgery is recommended. Non-pharmacological components of the management include promoting or changing lifestyle, such as engaging in regular exercise and avoiding a sedentary lifestyle (Cardel *et al*., 2020). Change in diet and nutritional counselling, avoiding processed food and diets with added sugars, eating a diet with more nutritional value rather than a diet that is more energy-concentrated (Cardel *et al*., 2020; Calcaterra & Zuccotti, 2022). However, in some cases, pharmacological intervention may be required to manage such conditions, and surgical intervention may be necessary in severe cases (Cardel *et al*., 2020).

In Pharmacotherapy for cardiometabolic diseases, the primary goal of the treatment approaches is to control individual risk factors, such as diabetes, hypertension, and dyslipidaemia (Ren & Zhang, 2018). In this approach, it may be necessary to prescribe

and administer several medications to a patient in order to increase life expectancy and improve quality of life (Seidu *et al*., 2023).

Examples of medications used in the treatment of cardiometabolic diseases includes: (Antwi, *et al*., 2024.; Patel *et al*., 2025)

- Lipid lowering agents, for example statins, such as atorvastatin and simvastatin
- Blood pressure lowering agents for example angiotensin converting enzyme inhibitors (e.g. Lisinopril) and calcium channel blockers (e.g. Amlodipine)
- Anti-diabetic medications exemplified by metformin, SGLT2 inhibitors (e.g. empagliflozin and dapagliflozin), GLP-1 receptor agonists (e.g. Liraglutide and semaglutide that facilitate insulin release, control blood sugar and decrease obesity).

Considering the different classes of drugs to be prescribed for the patient with cardiometabolic diseases, this might affect treatment outcome. It might be difficult to stick to the drug regimen, especially while taking several medications. The cost of medications, side effects, and discomfort in taking many medications are all factors that demerit treatment outcomes. It has also been reported that in multiple drug prescription, each drug added to prescription increases prescription error by about 16% (Seidu *et al*., 2023).

Surgical intervention; as obesity is considered to be a leading risk factor for CMD, in severe cases of obesity, metabolic and bariatric surgery is usually recommended as the most preferred surgical intervention (Cardel *et al.,* 2020).

Considering the complexity associated with the available conventional approaches to the treatment of cardiometabolic diseases, a better alternative that is more user friendly, affordable, accessible, and with no or minimal side effects, phytochemicals

have been shown to have potential in the treatment and prevention of cardiometabolic diseases (Rao, 2018).

2.4.1.2 Phytochemicals in the treatment of cardiometabolic diseases

Progress and advances have been made in the management and treatment of cardiometabolic diseases, but challenges still exist that warrant improvement and finding better ways to prevent such diseases and target those at risk (Sattar *et al.,* 2020). Despite the progress made in the treatment and management of these diseases, the incidence and prevalence of these diseases are still on the increase (Rao, 2018). This may not be unconnected with the nature of the treatment, treatment is sometimes expensive, accessibility may be difficult, associated with side/adverse effects leading to poor compliance, and some treatment modalities do not confer permanent cure, as such people prefer choices that are affordable, accessible, with fewer or no side effects (Rao, 2018).

Because people have a history of only attending healthcare facilities when they are unwell, efforts for disease prevention must be made, and one of the best times for CMD prevention is throughout adolescence, and to continue throughout life (Rao, 2018). Nutraceuticals such as naringin have been proposed for the treatment and prevention of CMDs. Nutraceuticals have been shown in studies to be promising in tackling the growing burden of CMDs and are projected to save massive healthcare spending, giving relief to both individuals and authorities (Bruno *et al.,* 2022). Naringin is one of the promising compounds for disease treatment and prevention (Yang *et al*., 2022).

2.5 Naringin

Naringin, a flavonoid phytochemical with chemical structure ‘4’, 5, 7-trihydroxy flavanone-7-rhamnoglucoside’, is a major component of grapefruit, tomato, and lots of

citrus fruits (Ho *et al*., 2000; Bharti *et al*., 2014;). Naringin has a molecular weight of 580.4g/mol (Alam *et al*., 2013). In 1928, the chemical/molecular structure of naringin (see Figure 2.2) was depicted by Asahina and Inubuse (Alam *et al*., 2020). Flavonoids are natural plant compounds available in vegetables and herbs (Stevens *et al*., 2019). The chemical structure of flavonoids (Figure 2.3) is made up of 15 carbon atoms and 3 rings, of which 2 belong to benzene rings connected with a 3-carbon chain, consisting of a flavonoid backbone, two phenolic rings, and a heterocyclic pyran ring (Shilpa *et al*., 2023). The structure is made of A, B, and C rings (Dias *et al*., 2021). The A ring originates from resorcinol or phloroglucinol, synthesised from the acetate pathway with characteristic hydroxylation patterns at the 5 and 7 positions, but the B ring starts from the shikimate pathway (D'Amelia *et al*., 2018). Naringin is abundant in grapefruit and is responsible for the bitter taste in most citrus fruits (Cavia-Saiz *et al*., 2010). In grapefruit, a high concentration of naringin has been reported at about 800mg/L (Rouseff *et al*., 1987), and 100mg of naringin is equivalent to the naringin provided by 1.5 servings of grapefruit juice or by one fruit, this is according to the United Kingdom (UK) Food Standard Agency (Chanet *et al*., 2012). The amount of naringin in commercially marketed citrus juices ranges from 50 to 1200 mg/L (Ho *et al.,* 2000).

Naringin is a common content of the human diet, available in many dietary products used by humans (Bharti *et al.,* 2014). As a flavonoid, naringin can only be taken in the diet as it cannot be produced by the body Sharma *et al*., 2011). Naringin is found in a variety of plant species and is one of the most extensively utilised active chemicals in Chinese herbal medicine (Ravetti *et al*., 2023).

It has been attributed with a wide variety of beneficial effects, such as antioxidant, anti-inflammatory, antilipidemic, cardioprotective, neuroprotective, hepatoprotective, and

so many other functions (Alam *et al.,* 2020; Benavente-García & Castillo, 2008; Chanet *et al.,* 2012; Shackebaei *et al*., 2023).

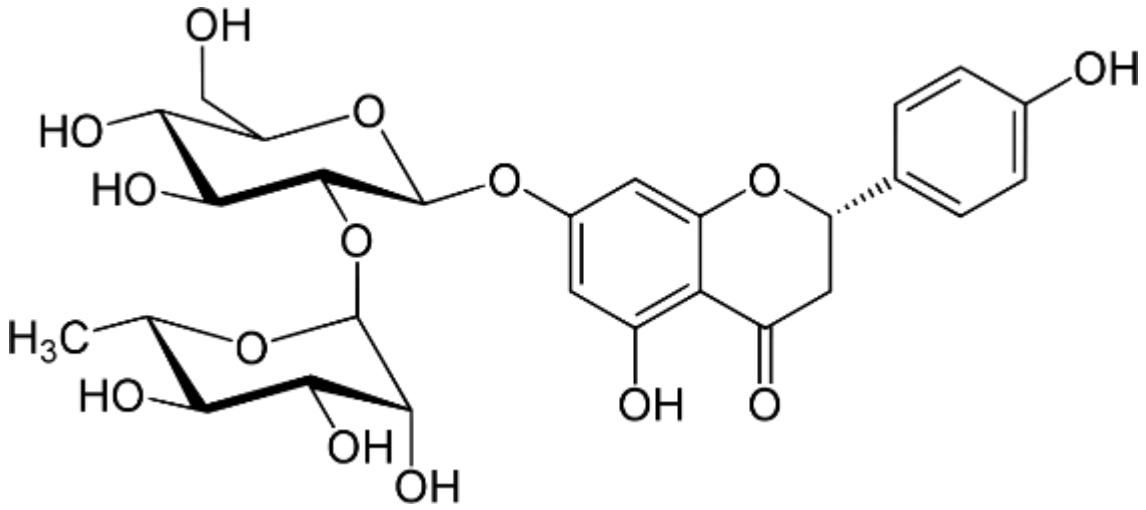

Figure 2.2: Chemical structure of Naringin (Ansicht, 1928)

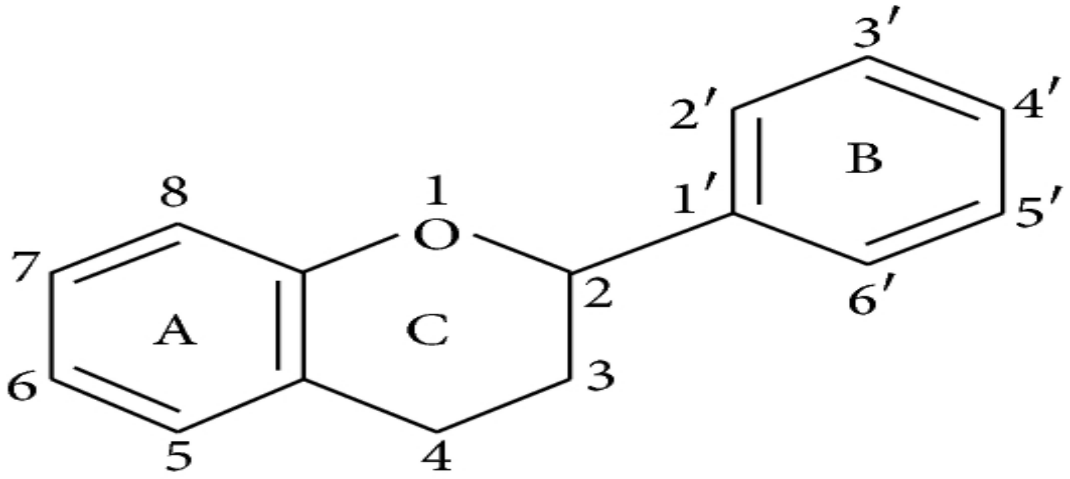

Figure 2.3: Chemical structure of Flavonoids

2.5.1 Pharmacokinetics of Naringin

Following oral administration, naringin, through active transport, is mostly concentrated in the liver and bile, where different enzymatic processes convert it into its metabolites such as naringenin, naringenin glucuronide, and naringin sulphate in the blood (Liu *et al.,* 2011). The enzymes α-rhamnosidases and β-Glucosidases participate in the

hydrolysis of naringin to naringenin (Felgines *et al*., 2000; Kim *et al*., 1998). Naringin can also be transformed by intestinal microflora into many types of phenolic resin acids because of ring fission (Kim *et al*., 1998). There are four major metabolites of naringin: 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, phloroglucinol, 4-hydroxyphenylpropionic acid, and 4-hydroxyphenylacetic acid (Kim *et al.,* 1998; Alam *et al.,* 2020).

Within five hours following oral administration, naringin is found in the plasma (Li *et al.,* 2004). Because Naringin and its metabolites are extremely lipophilic, they are transported to most bodily organs, with the highest concentrations found in the abdomen and the lowest in the brain due to reduced blood-brain barrier permeability (Alam *et al.,* 2020). A greater percentage of naringin is absorbed in the portal system (Tsai & Tsai, 2012), with a lesser part of it in the lymphatic system (Alam *et al.,* 2020).

Naringin is eliminated by the kidneys into the urine, and by the liver into digestive juice after partially undergoing bacterial ring cleavage (of the C-ring) and then the three carbon bridges to the dihydrochalcone moiety (Alam *et al*., 2020; Tsai, 2002). Naringin has a half-life of approximately 3 hours, with about 8% bioavailability (Mohamed *et al*., 2018). In a study by Liu and co-workers, the maximum plasma concentration of naringin was 6-8 hours at a dose of 50mg/kg/day and 6-8 hours at 250mg/kg/day, and 6-10 hours at a maximum dose of 1250mg/kg/day (Liu *et al*., 2011). Naringin has therapeutic effects on numerous health conditions due to its availability, affordability,

different pharmacological functions, and a prolonged period of use from a historical perspective (Chen *et al*., 2016).

Naringin shows no toxicity profile after acute, subacute, and chronic administration in Sprague-Dawley rats at 50, 250, and 1250mg/kg/day (Li *et al*., 2013, 2014). In

humans, naringin's oral no observed adverse effect level (NOAEL) is about 200 mg/kg (Li *et al.,* 2013).

2.5.2 Different pharmacological actions of naringin

2.5.2.1 Naringin as an antioxidant and anti-inflammatory agent

The major driver of the progress of many disease processes is inflammation, which is a component of vascular tissues' biological reaction to adverse stimuli such as pathogens, damaged cells, or irritants (Ferrero-Miliani *et al*., 2007). Inflammation initiates the pathogenesis of many cardiometabolic diseases such as atherosclerosis, heart diseases, obesity, metabolic syndrome, T2DM, and stroke (Rao, 2018). Obesity is marked by inflammatory mechanisms brought in by infiltration of the adipocytes by macrophages and mast cells (Weisberg *et al*., 2003; Xu *et al*., 2003). Adipose tissues also act as endocrine organs that produce inflammatory mediators/adipokines like IL-6, TNF-α, and leptin (Yu *et al*., 2006). These inflammatory mediators are believed to be in high proportion in individuals with metabolic derangements (Shoelson *et al*., 2007; Wisse, 2004). Tumour necrosis factor-α is the major inflammatory mediator in obese individuals, and it facilitates insulin resistance and damage to β-insulin-producing cells of the pancreas (Lin *et al*., 2012; Stephens *et al*., 1997). TNF-α disrupts insulin signalling by activating serine/threonine (ser/Thr) kinases that mediate the function of insulin receptors and insulin-receptor substrates, rendering them as non-functional substrates for insulin-mediated tyrosine phosphorylation (Yu & Ginsberg, 2005). Naringin has been shown in animal models of inflammation to be effective in reducing the expression of signalling factors associated with the inflammatory response, such as interleukin-6 (IL-6), interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS), nuclear factor erythroid 2-related factor 2 (Nrf2), and TNF-α (Chen *et al*., 2016). Habauzit *et al* show the ability of naringin to potentially halt an increase in serum IL-6 during

ageing-related inflammation in a 20-month-old male Wistar rat experimental model (Habauzit *et al*., 2011). As an anti-inflammatory flavonoid, naringin counteracts the increased level of TNF-α and improves inflammatory cell infiltration in an air-pouch model of inflammation (Jain & Parmar, 2011). In the LPS-induced TNF-α liver injury rat model, naringin supplementation resulted in decreased levels of TNF-α and prevented liver injury (Kawaguchi *et al*., 1999). Pre-treatment with naringin sufficiently ameliorates high-glucose-induced expression of cell adhesion molecules in human umbilical vein endothelial cells by suppressing a high glucose-induced increased expression of NF- kB (Lee *et al*., 2009).

In 3-nitro propionic acid-induced neurodegeneration rat models, naringin has been reported to cause upregulation of NADPH: quinone oxidoreductase 1, Heme oxygenase-1(HO-1), Placental Glutathione-S-transferase (GST p) and γ-glutamylcysteine ligase mRNA expression accompanied by activation of nuclear factor-erythroid 2-related factor (Nrf2). Through a similar mechanism, naringin causes decreased expression of pro-inflammatory mediators like TNF-α, cyclooxygenase 2, and induced nitric oxide synthase (iNOS) (Gopinath & Sudhandiran, 2012). Nrf2 facilitates the control of cellular antioxidant secretion, and this is significant in the mechanism of many degenerative diseases (Alam *et al.,* 2013). Naringin and naringenin are also associated with free radical scavenging and antioxidant ability and prevent lipid peroxidation (Cavia-Saiz *et al.,* 2010). Naringin sufficiently prevents the activity of xanthine oxidase, an enzyme that produces superoxide anions in the nucleus-containing cells (Russo *et al.,* 2000). Antioxidant enzymes such as SOD, catalase, and GPx were improved by naringin in diabetic rats, displaying a good protective function (Ali *et al.,* 2004; Jeon *et al.,* 2002; Punithavathi, 2008). In another study involving cholesterol-fed rabbits, naringin also proved its antioxidant effects by

maintaining the concentration of thiobarbituric acid reactive substances (TBARS) unchanged in rabbits fed a high-cholesterol diet after treatment with naringin (Jeon *et al.,* 2002). Also, in the heart of isoproterenol-induced Wistar rats, naringin shows an antioxidant property by preventing cardiotoxicity induced by isoproterenol (Rajadurai & Stanely Mainzen Prince, 2007).

2.5.2.2 Naringin in the management of obesity, dyslipidaemia, and metabolic syndrome

Metabolic syndrome is a conglomeration of different risk factors associated with cardiovascular disease, such as obesity, hypertension, insulin resistance, and NAFLD (Rochlani *et al.,* 2017). It is associated with poor dietary habits such as increased added sugar and fatty foods (Phillips & Prins, 2008). Naringin, through its anti-inflammatory effects, reduced blood lipid concentration, oxidative stress, and improved mitochondrial function and as such reduced obesity and risk of metabolic syndrome (Alam *et al.,* 2013; Kumar *et al.,* 2019). Many studies have been conducted, and findings are in line with the effects of naringin in converting and preventing obesity and dyslipidemia. High-fat diet supplemented with naringin in rats has shown an ability of naringin to lower the increased plasma lipids concentration and reduced lipid and cholesterol in high-cholesterol-diet-fed rats (Pu *et al.,* 2012). Naringin supplementation in low-density lipoprotein receptor (LDLR)-knocked mice was able to affect a reduction in the activity of HMG-CoA reductase significantly, and to increase the production of nitric oxide (NO) metabolites in the urine (Li *et al.,* 2004). Administration of naringin in rats were also able to enhance the expression of carnitine palmitoyl transferase-1 (CPT-1) and uncoupling protein-2 (UCP-2) which are regulated by Peroxisome proliferator-activated receptor alpha (PPAR α) (Cho *et al.,* 2011). A mouse with deficient Low-density lipoprotein receptors was fed a high-fat diet and displayed features of obesity and metabolic syndrome. In this study, Naringin administration was

able to prevent an increase in blood insulin and displayed a reduced hepatic sterol regulatory element binding protein (SREBP) -1c and hepatic lipogenesis. Decreased hepatic TG, leads to VLDL-TG formation and VLDL-apoB secretion and in that way mitigates dyslipidaemia (Mulvihill *et al.,* 2009). Naringin has also been shown to decrease plasma cholesterol, TG, and circulating free fatty acids (FFA) in high-fat/high-carbohydrate-diet-fed obese rats (Alam *et al.,* 2013). Hypercholesterolemic patients treated with naringin in a clinical trial have displayed decreased plasma LDL-c and HDL-c remained normal (Jung *et al.,* 2003). Naringin was able to decrease fat deposition in experimental rats by significantly increasing PPAR α, CPT-1, and UCP- 2 expression in the liver (Cho *et al.,* 2011). In adipose tissues, adipocyte differentiation is seen as a key factor in fatty deposition. This is facilitated by adipose tissue-derived MCP-1 that potentiates macrophage infiltration of adipose tissue and activates the release of inflammatory marker TNF-α that further worsens the inflammatory event (Yu *et al*., 2006). Naringin supplementation has been shown to block the production of MCP-1, TNF-α, and nitric oxide (NO) (Hirai *et al.,* 2007), through which naringin mitigates the inflammatory changes in adipose tissues (Alam *et al.,* 2014). Naringin also inhibits TNF-α mediated inflammation and tissue damage in the liver and vasculature. It also regulates AMPK, PPARa-, and CPT-1-mediated fat utilization and protects mitochondrial function in metabolic syndrome, obesity, and concomitant cardiovascular diseases (Alam *et al.,* 2014). Naringin, therefore, improves metabolic syndrome by increasing AMPK activity and decreasing the expression of major gluconeogenic enzymes. It also decreases HMG-CoA reductase activity while increasing NO metabolite synthesis (Chen *et al.,* 2016). The antioxidant properties of naringin are achieved through the activation of Nrf2, a transcription factor that plays a central role in cellular defence against oxidation (Gopinath & Sudhandiran, 2012). Naringin lowers the expression of pro-inflammatory mediators such as tumour

necrosis factor-alpha, cyclooxygenase-2, and inducible nitric oxide synthase and increases the expression of NAD(P)H: quinone oxidoreductase-1, haem oxygenase-1, glutathione S-transferase P1, and gamma-glutamylcysteine ligase mRNA (Gopinath & Sudhandiran, 2012).

2.5.2.2 Naringin as a hypoglycaemic/anti-diabetic agent

Hyperglycaemia is the increase in the concentration of glucose within the blood. It is believed to be initiated when there is decreased peripheral cell sensitivity to insulin, reducing glucose utilisation by the cells and causing glucose buildup in the blood (Alam *et al.,* 2013). Both mitochondrial and cytosolic ROS are produced more readily in hyperglycaemia, and the oxidative stress that follows leads to the development of numerous clinical problems associated with diabetes (González *et al*., 2023). Insulin resistance and diabetes mellitus (DM) facilitate the increased release of inflammatory mediators such as interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α) (Kado *et al*., 1999; Pickup *et al*., 2000). TNF-α and IL-6 are associated/implicated in the aetiology of insulin-receptor dysfunction, promoting insulin resistance and hyperglycaemia (Dandona *et al*., 2004; Krogh-Madsen *et al*., 2006). A diet rich in fat also induces the secretion of inflammatory mediators, causing insulin resistance and increased blood glucose concentration(Lee *et al*., 2009; Terra *et al*., 2009). In a streptozotocin-induced diabetic rat model, naringin co-administered with vitamin C, prevented diabetes by increasing blood levels of insulin and improving oxidative stress (Punithavathi, 2008). Naringin supplementation was also shown to improve insulin concentration and prevent damage to the pancreatic cells in mice (Jung *et al*., 2004). Naringin significantly decreased the activity of hepatic glucose-6-phosphatase and phosphoenol pyruvate carboxy kinase in db/db mice (Jung *et al.,* 2004). Naringin also facilitates glucose uptake in the skeletal muscle via the upregulation of AMPK in the cells of skeletal muscle (Zygmunt *et al*., 2010). Additionally, in a high-fat diet mouse

model, naringin administration ameliorated glucose intolerance and insulin resistance (Pu *et al*., 2012). In diabetes-induced male rats, naringin administration at 50mg/kg lowered blood glucose and plasma glycated haemoglobin (HbA1c) levels while increasing insulin levels, and oxidative stress-induced inflammatory markers such as IL-6, TNF-α, and NO were also decreased after naringin supplementation. Naringin also increased the antioxidant enzyme activity and the levels of glutathione (Mahmoud *et al*., 2012).

2.5.2.3 Naringin as a hepatoprotective agent

Many research findings have documented the hepatoprotective effects of naringin. In a nickel and cadmium-induced liver toxicity rat model, naringin administration sufficiently decreased the activity of increased plasma transaminase(Pari & Amudha, 2011; Renugadevi & Prabu, 2010). In the liver, naringin has been shown to decrease lipid peroxidation and increase the activity of SOD, catalase, GPx, and GST, which provides antioxidant defence against liver damage (Pari & Amudha, 2011; Renugadevi & Prabu, 2010). In a rat model of dimethyl nitrosamine-induced liver toxicity, naringenin supplementation improved serum albumin and total protein concentration and decreased malondialdehyde levels in the liver (Lee *et al*., 2004)(Lee *et al.,* 2004). Within the liver of fructose-fed rats, naringin administration sufficiently decreased oxidative and nitrosative stress compared to control (Kannappan *et al*., 2010). Likewise, it reduced fat accumulation in the liver in a high-fat diet rat model (Pu *et al.,* 2012). Lipopolysaccharide (LPS) facilitates liver injury by its ability to induce the release of TNF-α. Naringin prevents LPS-induced liver injury by decreasing the secretion of TNF-α (Kawaguchi *et al*., 1999). In a high-fat diet diabetic male albino rat model, naringin supplementation facilitated the expression of PPAR-Ɣ in the hepatocytes and reduced expression of liver X receptor (LXR), SREBP-1c, and SREBP-1a in a fatty liver (Sharma *et al.,* 2011).

High-carbohydrate, high-fat diet obese rats show the decreased activity of liver enzymes makers such as Aspartate Aminotransferase (AST), Alanine transaminase (ALT), and Alkaline phosphatase (ALP) and decreased fatty accumulation and liver fibrosis with naringin supplementation (Alam *et al.,* 2013).

2.5.2.4 Naringin as a cardioprotective agent

Naringin is a well-known cardioprotective agent with therapeutic and preventive potential against different cardiovascular conditions. This has been possible because of its antioxidant and anti-inflammatory properties (Yaseen *et al*., 2024).

In rats with sepsis-induced myocardial dysfunction, naringin demonstrated a protective effect by reducing lipopolysaccharide-induced cardiac oxidative stress through the Kelch-like ECH-associated protein 1/Nuclear factor erythroid 2-related factor/Heme oxygenase-1 (Keap1/Nrf2/HO-) pathway (Sun *et al*., 2021). Naringin improved left ventricular function and reduced interstitial fibrosis and cardiac hypertrophy in pressure-overloaded mice (Zhang *et al.,* 2015). Naringin's known antioxidant properties may be responsible for its ability to reduce cardiac fibrosis via modifying the expression of the p38 and Protein Kinase C beta (PKC-β) proteins (Adebiyi *et al*., 2016). Naringin reduced diastolic calcium overload, enhanced glucose transport, decreased the generation of reactive oxygen species, and inhibited myocardial inflammation to protect against type 2 diabetes-induced cardiomyocyte injury and improved diastolic dysfunction (Uryash *et al*., 2021). Naringin administration has been shown to decrease LV hypertrophy, LV fibrosis, and LV stiffness by reducing inflammatory and oxidative stress. It also facilitated the recovery of some left ventricular variables like fractional shortening and ameliorated cardiac remodelling in the heart of an experimental rat (Alam *et al.,* 2013). In high cholesterol-fed rats, naringin administration improved endothelial dysfunction and LV hypertrophy

(Pengnet *et al*., 2019). By inhibiting reactive oxygen species-dependent Ataxia-Telangiectasia Mutated-mediated (ROS-dependent ATM-mediated) P53 signalling (Park *et al*., 2018), and oxidative stress (Visnagri *et al*., 2015) naringin protected against fructose-induced cardiac hypertrophy and improved ventricular dysfunction. Naringin has been shown to inhibit myocardial fibrosis, ameliorate features of LV systolic and diastolic dysfunction in rats with chronic heart failure (Ruan *et al.,* 2024).

By controlling the AMPK-mTOR signalling axis, naringin decreased the formation of ROS and mitochondrial dysfunction in cardiomyocytes exposed to fructose, which in turn decreases cardiomyocyte hypertrophy (Park *et al.,* 2018) and acute myocardial infarction (Chen *et al.,* 2016).

Aside from the cardiometabolic potential associated with naringin, naringin also has preventive and therapeutic potential in other disease conditions.

2.5.2.5. Naringin in other disease conditions

Through a variety of signalling channels and molecular processes, naringin significantly contributes to the anti-inflammatory and antioxidant regulation of different systemic diseases (Peng *et al.,* 2024).

In an acute kidney injury model, naringin was shown to protect the kidney via modifying microRNA-10a in the renal tissue (Amini *et al*., 2022). Through inhibiting the MAPK/ NF-κB inflammatory signalling pathway and promoting the activation of the Nrf2 antioxidant signalling pathway, naringin ameliorated severe lung injury caused by *Actinobacillus pleuropneumoniae* in a mouse model (Huang *et al*., 2024). For several neurological disorders, such as Alzheimer's Disease, Parkinson's Disease, cerebral ischemia, anxiety, depression, schizophrenia, chronic hyperglycaemia, and peripheral neuropathy, naringin and naringenin have shown to have a treatment potential (Emran

et al., 2023). Naringin and naringenin inhibit the growth of cancerous cells and prevent their spread. As a result, they have intriguing therapeutic potential for use as efficient substitute treatments for cancer patients. Because both substances can overcome resistance to traditional chemotherapy and boost the effectiveness of chemotherapeutic medicines, they may be utilised as an adjuvant therapy (Stabrauskiene *et al*., 2022). In an osteoporotic SD rat model, naringin showed the potential to promote osteogenesis. It also improved the bone weight index, length, and diameter of the rat femoral bone (Zhao *et al*., 2016). The gastroprotective effect of naringin was demonstrated in rats after acetylsalicylic acid (ASA)-induced peptic ulcer (Galati *et al*., 1998), and diclofenac sodium-induced peptic ulcer disease.

Even though naringin has been shown to possess various therapeutic and preventive potentials against different cardiometabolic indices as discussed above, the potential of naringin against sweetened alcohol-induced cardiometabolic derangements has not been reported, and more research needs to be done, which is the focus of this study.

Having highlighted the burden of alcohol consumption, the increased popularity and consumption of sweetened alcohol, especially among the younger population, the relationship between sweetened alcohol consumption and heavy or inappropriate alcohol consumption, the increased double burden of sweetened alcohol consumption, and risk of cardiometabolic diseases, and the potential therapeutic effects of naringin in the treatment and prevention of sweetened alcohol-induced cardiometabolic dysfunctions. The following research gaps have been identified:

i. Previous studies focused more on the individual effects of alcohol and fructose
ii. Previous studies emphasised more on male subjects

iii. Previous studies used a forced alcohol consumption model in animals instead of a more realistic voluntary consumption model
iv. Protective potentials of naringin against sweetened alcohol-induced cardiometabolic derangements have not been explored.

The focus of this thesis is on three target organs that are most impacted by alcohol consumption and high fructose intake, namely the heart, liver and kidneys. In the next chapter, the impact of sweetened alcohol and naringin on cardiovascular structure and function is explored. The chapter is presented in a word format in the publication.

CHAPTER THREE - EFFECTS OF VOLUNTARILY CONSUMED SWEETENED ALCOHOL AND NARINGIN ON CARDIAC FUNCTION IN MALE AND FEMALE SPRAGUE-DAWLEY RATS

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3.1 Abstract

This study assessed the impact of sweetened alcohol and naringin on cardiac function in Sprague-Dawley rats. Male (n = 40) and female (n = 40) rats were allocated to control, sweetened alcohol (SOH), naringin (NA), and sweetened alcohol with naringin (SOH + NA) groups. SOH and SOH + NA rats received 10% alcohol + 20% fructose in gelatine; SOH + NA and NA rats received 50 mg/kg naringin in gelatine daily for 10 weeks. Echocardiography was performed to assess left ventricular (LV) function. LV cardiomyocyte diameters and collagen area fraction were determined by H&E and picrosirius-red staining, respectively. In males, sweetened alcohol and naringin did not affect cardiac function. Female SOH rats had increased LV end-diastolic posterior wall (p = 0.04), relative wall thicknesses (p = 0.01), and LV cardiomyocyte diameters (p = 0.005) compared with controls. Female SOH and SOH + NA had reduced lateral e' and e'/a' and increased E/e' (p < 0.0001). Female SOH (p = 0.01) and SOH + NA (p = 0.04) rats had increased LV collagen area fraction compared with controls. In males, neither sweetened alcohol nor naringin affected cardiac geometry or diastolic function. In females, sweetened alcohol induced concentric remodelling, impaired LV relaxation, and elevated filling pressures. Naringin may have the potential to improve the sweetened alcohol-induced concentric remodelling; however, it did not ameliorate diastolic dysfunction in females.

Keywords: cardiac geometry, diastolic function, naringin, sweetened alcohol

3.2 Introduction

Modern society has seen a rise in the popularity of sweetened alcoholic beverages, including flavored spirits, jello shots, and cocktails (Crews, 2015; Mosher & Johnsson, 2005; Wakabayashi *et al*., 2021). The sweeteners incorporated into these beverages mask the presence of alcohol, making the beverages more palatable and consequently less apparent how much alcohol is being ingested. This can increase the risk of overconsumption, particularly among adolescents who often start drinking these beverages at a young age (Binakonsky *et al*., 2011; Crews, 2015; Mola *et al*., 2023). Common sweeteners in these beverages typically include sucrose (a blend of 50% glucose and 50% fructose), high fructose corn syrup, or concentrates from fruit juice (primarily fructose) (Poppitt, 2015). The combination of alcohol and fructose in these drinks poses a double health risk burden, as individually, alcohol and fructose have been associated with negative cardiometabolic health outcomes such as obesity, dyslipidaemia, and insulin resistance (Poppitt, 2015). These modifiable risk factors may lead to the development of cardiovascular diseases such as heart failure and atherosclerosis (Liu *et al*., 2022; Meijers & De Boer, 2019; Tan *et al*., 2020). A high fructose diet may cause cardiac inflammation and fibrosis through various mechanisms including increased production of pro-inflammatory factors, upregulation of pro-fibrotic gene expression, macrophage infiltration, and heightened oxidative stress (Kang *et al.,* 2016; Wang *et al.,* 2023; Xu *et al.,* 2022; Zhang *et al.,* 2016). Alcohol consumption may result in increased reactive oxygen species (ROS) leading to reduced endothelial nitric oxide synthase in cardiomyocytes (Tsermpini *et al*., 2022; Wang *et al*., 2015). Therefore, the combined effects of fructose and alcohol may cause several structural changes as well as functional disturbances in the heart which may predispose individuals to cardiac dysfunction. Although fructose and alcohol

individually have been extensively studied for their effect on cardiac function, their combined effects remain an area of ongoing investigation. Managing the cardiometabolic consequences of alcohol and high fructose consumption results in a heavy socioeconomic burden for many countries globally (Rao, 2018). There is a need to explore prophylactic interventions early in life to reduce the poor health outcomes associated with alcohol and sugar consumption. Phytochemicals are increasingly being targeted as candidates for nutraceutical potential (Bruno *et al*., 2022).

Naringin, a flavonoid phytochemical found in citrus fruit, has recently become of interest for its possible cardioprotective effects (Akin *et al.,* 2022; Malakul & Pengnet, 2018; Shackebaei *et al.,* 2023; Uryash *et al.,* 2021). Studies have reported that naringin has antioxidant and anti-inflammatory properties (Akin *et al.,* 2022; Uryash *et al.,* 2021) that may protect the myocardium by reducing inflammation as well as oxidative stress that takes place after the consumption of fructose or alcohol. Nevertheless, to the best of our knowledge, there is no literature regarding the effects of naringin on sweetened alcohol-induced cardiac changes. Consequently, further studies are required to determine naringin's potential cardioprotective effects in the context of sweetened alcoholic beverage consumption. There is a bias towards the use of males in animal-based studies, despite the societal trends showing that both males and females consume sweetened alcoholic beverages (Wakabayashi *et al.,* 2021). Therefore, this research aimed to investigate the effects of sweetened alcohol and naringin on cardiac function in male and female Sprague–Dawley rats.

3.3 Methods

3.3.1 Ethical clearance and study site

The study adhered to international ethical guidelines and standards governing the care and utilization of laboratory animals, receiving approval from the Animal Research

Ethics Committee of the University of Witwatersrand (AREC clearance certificate number 2022/10/02/C). The research took place at the Wits Research Animal Facility (WRAF) situated at the University of the Witwatersrand in Johannesburg, South Africa. The study adhered to the Animal Research Reporting of *in Vivo* Experiments (ARRIVE) guidelines both during the research process and in the compilation of the manuscript.

3.3.2 Animals and study design

A total of 80, 42-day-old (adolescent) Sprague–Dawley rats (comprising 40 males and 40 females) were sourced from the WRAF of the University of the Witwatersrand, Johannesburg. Each rat was housed in a perspex cage topped with steel mesh covers. The cages were lined with wood shavings and shredded paper for bedding and environmental enrichment.

Additional environmental enrichment included wooden blocks for the rats to gnaw on and sections of wide bore pipes for the rats to hide in. Rats were provided with ad libitum access to standard pelletized rat chow (LabChefR, Johannesburg, South Africa) and tap water for the entirety of the study. To maintain a hygienic environment, cages and bedding were changed twice a week. The room's ambient temperature was controlled at 26°C ± 2°C, with appropriate ventilation and a 12-h light/dark cycle (lights on at 07.00 h). The experiment was designed as a randomised, interventional, prospective study. The rats were subjected to a 1-week acclimatization period wherein initial assessments, including body mass, food consumption, and blood pressure, were recorded. The rats were also acclimatized with gelatine, which was prepared by adding 0.5 g fructose with 6 g gelatine in 100 mL of water. It was given daily at 10 mL/100 g body weight for all the rats during the acclimatization period. Following this

acclimatization period, rats were randomly assigned to one of four treatment groups, each consisting of 20 rats (10 males and 10 females):

1. Control: received plain gelatine (0.5% fructose in 6% gelatine)—fructose was added to the gelatine to improve the palatability of the preparation.
2. Sweetened alcohol (SOH): received sweetened alcohol (10% alcohol +20% fructose) in 6% gelatine.
3. Naringin (NA): received 50 mg/kg body mass (BM) naringin in gelatine (0.5% fructose in 6% gelatine).
4. Sweetened alcohol + naringin (SOH + NA): received sweetened alcohol (10% alcohol +20% fructose) + 50 mg/kg BM naringin in 6% gelatine.

The gelatine with or without alcohol and/or naringin was provided daily to the rats in glass containers at a volume of 10 mL per 100 g body mass. The use of gelatine for voluntary administration of alcohol has been previously described and reported in various studies (Dess *et al.,* 2013; Peris *et al*., 2006; Ralevski *et al*., 2006). Before the commencement of the intervention, baseline blood pressure and body masses were measured. The study design is depicted in Figure 3.1 below.

3.3.3 Interventions used and preparation

For our study, gelatine (Sheridan's gelatine, Libstar Operations (Pty) Ltd, Dunkeld, South Africa) was used as a vehicle. Plain gelatine was prepared by mixing 6 g gelatine with 0.5 g fructose powder (Fructose, Nature's Choice, Randvaal, South Africa), fructose administered to SD-rats at a milder dose (5.3g/kg/day) for 20 weeks does not affect rat's metabolism (Song *et al*., 2021) and 1–2 mL Bovril (Beefy Bovril, Pioneer Foods (Pty) Ltd, South Africa) in 100 mL of hot water. Naringin was incorporated into gelatine by adding 0.05 g (to provide a dose of 50 mg/kg body mass (Adebiyi *et al.,* 2016), naringin (>95% HPLC, CAS No. 10236-47-2, Sigma-Aldrich,

China) to the plain gelatine as described above. Sweetened alcohol in gelatine was prepared by adding 20 g fructose powder (equivalent to 20% fructose suspension (Komnenov *et al*., 2020), 6 g gelatine, and 1–2 mLBovril, with 10 mL of ethanol (equivalent to 10% ethanol (Al-Awwadi *et al.,* 2004) (Ethanol 99.9%; CAS No. 64-17-5; Batch No. 37500; ACE chemicals, South Africa) to provide up to a maximum ethanol dose of 0.79 g/100 g body weight, in 90 mL of hot water (<60°C). The alcohol used has a purity of 99.9% according to the manufacturers (other constituents that make the 0.1% includes acetic acid, acetaldehyde, esters, iron, lead, methanol, and water). This dose is equivalent to about half of a glass/unit of alcohol in humans which corresponds to chronic light alcohol consumption (Brick, 2006) and has been associated with elevated liver enzymes (Niemelä *et al*., 2017). Sweetened alcohol with naringin was prepared by adding 0.05 g naringin (to provide a dose of 50 mg/kg body mass) of naringin into the sweetened alcohol in gelatine preparation as described above (doses were adjusted to the rats' body mass). In each case, the mixture was thoroughly mixed using a magnetic hot plate stirrer. The solutions/mixtures were then placed into glass containers and sealed to prevent evaporation of the alcohol. All preparations were kept and allowed to set in a fridge at 2–7°C for 4–24 h before being served to the rats. All treatments (either plain gelatine, sweetened alcohol, naringin in gelatine, or sweetened alcohol with naringin) were offered to the rats once daily (at approximately 2:00 pm) at a dosage of 10 mL per 100 g body mass over 10 weeks. The gelatine preparation consumed by each rat was estimated by subtracting the remaining gelatine in millilitres (mL) from the gelatine provided each week. Relative gelatine consumption was determined by dividing the gelatine consumed by body mass and then multiplying by 100.

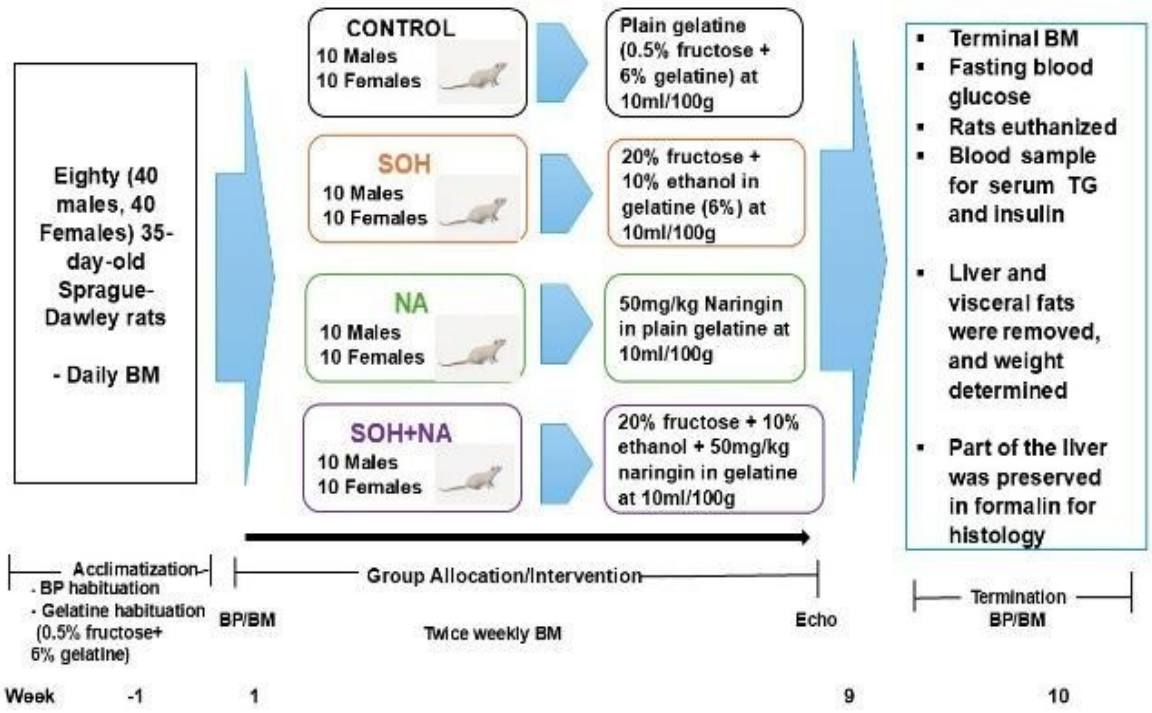

Figure3.1: Schematic diagram of the study design

BP: Blood pressure; BM: Body mass; Echo: Echocardiography; LV: Left ventricle; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol + naringin

3.3.4 Body mass measurements

An electronic weighing balance (Snowrex EQ-1200, Snowrex International Company, Taipei, Taiwan) was used to measure the baseline body mass. Subsequently, measurements were conducted twice weekly to assess growth progress and make necessary adjustments to the dosage of fructose, gelatine, ethanol, and naringin, thereby ensuring a consistent dosage proportionate to the body mass. The final body mass was measured at termination.

3.3.5 Non-invasive blood pressure measurements

Blood pressure was measured at baseline and termination using the tail-cuff technique (Biopac Systems, Santa Barbara, CA, USA), as previously described (Pauline *et al*., 2011). Briefly, each rat (priorly habituated to the procedure) was put in a restrainer,

with the rat's tail on a heating pad. After the rats' tails were heated, the blood pressure machine's cuff was placed at the base of the tail, and readings of blood pressure were recorded. Two to three readings per rat were taken, and the average was determined. To prevent diurnal variation, measurements were done at midday.

3.3.6 Echocardiography measurements

Nine weeks after the commencement of the intervention, rats were anaesthetised with isoflurane delivered in 100% oxygen using a precision vaporiser with an induction chamber and a nosecone for maintenance. Induction was performed at 3–4% isoflurane with an oxygen flow of 2–3 L/min until loss of the righting reflex (≈1–3 min). For echocardiography, anaesthesia was maintained at 1.0–1.5% isoflurane with an oxygen flow of 0.5–1.0 L/min to preserve heart rate and myocardial function. The anterior chest wall was shaved. Echocardiography was performed with the rats in the left lateral decubitus position using a high-resolution ultrasound probe (10 MHz) coupled to an echocardiographic machine (Affiniti CVx, Philips Healthcare, Andover, Massachusetts). LV dimensions were determined using two-dimensional directed M-mode echocardiography in the parasternal long-axis view during three consecutive beats. LV end-systolic and end- diastolic internal diameters, interventricular septal thickness, and posterior wall thickness was measured in systole and diastole. Relative wall thickness was calculated as (2 × posterior wall thickness)/(LV internal diameter at end-diastole) (Lang *et al.,* 2015). LV pump and myocardial systolic function were determined by calculating LV ejection fraction using the Teichholz method and LV endocardial fractional shortening (Lang *et al*., 2015). LV diastolic function was determined from the mitral valve inflow patterns using pulsed Doppler imaging. In the apical 4-chamber view, the early (E) and late (A) diastolic inflow velocities were obtained with the sample

volume placed at the mitral valve leaflet tip and expressed as E/A, a marker of relaxation. To determine diastolic function using tissue Doppler imaging (TDI), peak myocardial tissue lengthening velocities during early (e') and late (a') diastole were recorded at the lateral mitral annulus in the apical four-chamber view. Diastolic function markers are expressed as e' (an index of myocardial relaxation), e'/a' (an index of myocardial stiffness), and E/e' (an index of LV filling pressure). The rats were allowed to recover from the anaesthetic and then returned to their cages and respective treatments for a week. Measurements were taken in the LV since the LV plays a crucial role in ensuring blood circulates to all parts of the body. Given its thicker wall compared to the right ventricle, the LV is more often susceptible to various cardiovascular conditions, and therefore, studying the left side of the heart provides meaningful information about the overall cardiac function (Kerfoot *et al*., 2019). Moreover, cardiovascular diseases such as heart failure, myocardial infarction, and hypertension predominantly affect the left side of the heart (Tsao *et al*., 2015). Therefore, focusing on the left side provides a better understanding of the mechanisms associated with these conditions.

3.3.7 Terminal procedures

At the end of the 10-week intervention, rats were euthanised using a sodium pentobarbital overdose (Euthapent, Kyron laboratories, Johannesburg, South Africa) at 150 mg/kg BM injected intraperitoneally. Visceral fat (adjoining the liver, kidneys, pancreas, stomach, and small and large intestines) was carefully removed, and weights were determined using a Presica 310 M digital scale (Precision Instruments, Johannesburg, South Africa). The hearts were dissected out and their respective weights were recorded. Subsequently, the left ventricle was isolated from the other

cardiac structures, and its specific weight was determined using a Presica 310 M digital scale (Precision Instruments, Johannesburg, South Africa).

3.3.8 LV cardiomyocyte diameter and total collagen content determination

LV tissue was preserved in 10% phosphate-buffered formalin and later histologically examined. For the histological examination, the samples were processed using the Thermo Scientific's Microm STP 120 (Microm STP 120, Thermo Scientific, MA, USA) automatic tissue processor and embedded in paraffin wax. Processed tissues were sectioned at 4 µm thick using a microtome (Leica Biosystems Instruments (Pty), Ltd, model RM 2125, Wetzlar, Germany) and placed onto glass microscope slides. The slides were allowed to air-dry and adhere to a 20°C heating block.

3.3.9 Hematoxylin and eosin stain

The Hematoxylin and Eosin (H&E) stain was used to assess LV cardiomyocyte diameter (width) at the level of the nucleus in longitudinal sections. Briefly, the samples underwent deparaffinization with xylene, rehydration through a series of graded ethanol concentrations, and staining with Harris' hematoxylin followed by alcoholic eosin solution. Subsequently, the slides were washed (dehydrated) with ethanol and xylene before being mounted with DPX mountant and covered with a coverslip (Grosset *et al*., 2017). The slides were visually analyzed by a blinded evaluator using a Zeiss Axioskop 2 Plus microscope, equipped with a Zeiss AxioCam (Zeiss, Peabody, MA, USA), to analyze the LV cardiomyocyte diameter. Tissue sections viewed under a bright field were obtained using a 4× objective lens (×400 magnification). The LV cardiomyocyte diameters at the level of the nucleus in longitudinal sections were determined using the straight-line tool on ImageJ software (ImageJ version 1.54d, Laboratory for Optical and Computational Instrumentation, University of Wisconsin) (Baudouy *et al*., 2017).

LV cardiomyocyte diameters were measured three times per sample, and an average was determined.

3.3.10 Picrosirius red stain

The picrosirius red stain was used to measure the accumulation of collagen fibres to quantify the degree of fibrosis in LV samples. Briefly, sections were deparaffinized, rehydrated, and stained with a 0.1% Sirius Red solution dissolved in aqueous saturated picric acid for 60 min at room temperature. After washing in acidified water (5 mL of acetic acid into 1 L of distilled water), the slides were dehydrated and mounted with DPX mountant (Merck Life Science (Pty) Ltd, Modderfontein, South Africa) (Junqueira *et al*., 1979). The tissue sections were visually analyzed by a blinded evaluator using a Zeiss Axioskop2 Plus microscope equipped with a Zeiss AxioCam (Zeiss, Peabody, MA, USA). Tissue section images viewed under bright-field and polarized light were obtained using a 10× objective lens (x100 magnification). The collagen area fraction and overall tissue area for each tissue section were determined using ImageJ software (ImageJ version 1.54d, Laboratory for Optical and Computational Instrumentation, University of Wisconsin). Collagen area fractions were determined three times per sample, and an average was determined. The collagen area fraction was computed by dividing the collagen area by the overall tissue area (Baidoo *et al*., 2022).

3.3.11 Statistical analysis

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Graphs were generated using GraphPad Prism version 9.4 (GraphPad Software Inc., San Diego, CA, USA). The sample size was determined using the formula (Charan & Kantharia, 2013): 2 SD2 (1.96 + 0.842)2/d2 where SD =

standard deviation; 1.96 = type 1 error of 5%; 0.842 = at 80% power; 0.842 is a value from the standard formula used to determine the sample size. The value (0.842) is associated with the Z-score, or critical value related to achieving 80% power in a standard normal distribution. When using a standard normal distribution, a Z-score of approximately 0.842 corresponds to 80% power (Charan & Kantharia, 2013), and d = difference between mean values. Shapiro–Wilk tests were used for normality tests. Data were normally distributed and are expressed as means ± SEM. To determine differences in baseline and terminal body masses and blood pressure measurements, as well as weekly gelatine consumption measures, repeated measures two-way analysis of variance (ANOVA) was used with group and sex as the main effects. If significant differences were observed, Tukey post hoc tests were employed to identify specific group and time differences. Cardiac geometry and functional differences were assessed using two-way ANOVA with group and sex as the main effects. If significant differences were observed, Tukey post hoc tests were employed to identify specific group differences. Associations between tissue Doppler indices and the collagen area fraction were determined using Pearson's correlation. Since blood pressure contributes to the development of diastolic dysfunction, blood pressure was included as a confounder in the correlation analysis. $p < 0.05$ was considered significant.

3.4 Results

3.4.1 Morbidity and mortality

One of the male rats died from the Male SOH+NA group during the study due to iatrogenic causes (confirmed by autopsy).

3.4.2 Body mass, visceral fat mass, and blood pressure measurements

Table 3.1 shows the effects of sweetened alcohol and naringin on body mass and blood pressure at baseline and at termination in male and female Sprague–Dawley

rats. The group and sex effects were significantly different for baseline body mass (group: $F = 2.49$, $p = 0.02$; sex: $F = 13.27$, $p = 0.0005$) and terminal body mass (group: $F = 175.60$, $p < 0.0001$; sex: $F = 1208.68$, $p < 0.0001$). Body masses were significantly higher ($p < 0.0001$) at termination compared to baseline across all groups in both male and female rats. Males were heavier ($p < 0.0001$) compared to females at baseline and termination. The group and sex effects were significantly different for visceral fat mass indexed to body mass (group: $F = 16.44$, $p < 0.0001$; sex: $F = 76.13$, $p < 0.0001$). Female SOH and SOH + NA rats had heavier visceral fat masses indexed to body mass compared to female control and NA rats ($p < 0.0001$). Female SOH and SOH + NA rats had heavier visceral fat masses indexed to body mass compared to all male groups ($p < 0.0001$). There were no significant differences observed in group and sex effects for baseline systolic blood pressure (group: $F = 0.42$, $p = 0.89$; sex: $F = 0.13$, $p = 0.72$) and terminal systolic blood pressure (group: $F = 0.81$, $p = 0.58$; sex: $F = 0.11$, $p = 0.74$). There were no significant differences observed in group and sex effects for baseline diastolic blood pressure (group: $F = 1.13$, $p = 0.31$; sex: $F = 0.02$, $p = 0.92$) and terminal diastolic blood pressure (group: $F = 1.15$, $p = 0.34$; sex: $F = 0.00$, $p = 0.96$).

Table 3.1: Effects of sweetened alcohol and naringin on body mass, visceral fat mass, and blood pressure at baseline and termination in male and female Sprague-Dawley rats

	Males				Females			
	Control	SOH	NA	SOH+NA	Control	SOH	NA	SOH+NA
Baseline								
Body mass (g)	148 ± 6δ	144 ± 6δ	154 ± 6δ	156 ± 6δ	118 ± 6δ*α*	122 ± 6δ*α*	111 ± 6δ*α*	132 ± 6δ*α*
SBP (mm Hg)	116 ± 4	119 ± 2	124 ± 3	118 ± 4	119 ± 3	120 ± 3	120 ± 3	122 ± 3
DBP (mm Hg)	79 ± 2	75 ± 3	72 ± 2	76 ± 3	75 ± 3	78 ± 3	75 ± 3	79 ± 2
Termination								
Body mass (g)	463 ± 6	435 ± 6	460 ± 6	428 ± 6	258 ± 6*α*	271 ± 6*α*	257 ± 6*α*	268 ± 6*α*
Visceral fat (% body mass)	2.79 ± 0.38	2.74 ± 0.38	2.87 ± 0.41	2.87 ± 0.41	3.97 ± 0.38	6.17 ± 0.38*#*α*	4.09 ± 0.38	6.64 ± 0.38*#*α*
SBP (mm Hg)	117 ± 1	115 ± 3	119 ± 3	117 ± 2	119 ± 3	117 ± 2	117 ± 2	119 ± 3
DBP (mm Hg)	79 ± 2	79 ± 2	76 ± 2	78 ± 3	78 ± 2	79 ± 1	81 ± 1	77 ± 1

Data expressed as mean ± SEM. *p<0.05 compared to control. #p<0.05 compared to NA. δp<0.0001 compared to terminal body mass. αp<0.0001 compared to all male groups. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin.

3.4.3 Gelatine consumption

Figure 2 shows the effects of sweetened alcohol and naringin on weekly absolute and relative gelatine consumption in male and female Sprague–Dawley rats. The group and sex effects were significantly different for absolute gelatine consumption (group: $F = 54.81$; $p < 0.0001$; sex: $F = 365.25$; $p < 0.0001$). There were no significant differences in absolute gelatine consumption between the groups in both males and females for all 10 weeks (Figure 3.2A, C). Male rats had higher absolute gelatine consumption compared to females from week 4 to week 10 ($p < 0.01$; Figure 3.2A, C). When gelatine consumption was computed relative to body mass, the group effect was significant ($F = 11.78$; $p < 0.0001$); however, the sex effect was not ($F = 0.48$; $p = 0.48$). There were no significant differences in relative gelatine consumption between the groups in males for all 10 weeks (Figure 3.2D). In females, the relative gelatine consumption was higher in the control and NA groups compared to the SOH + NA group at week 8 ($p = 0.02$ and $p = 0.03$) and week 9 ($p = 0.03$ and $p = 0.03$; Figure 3.2D).

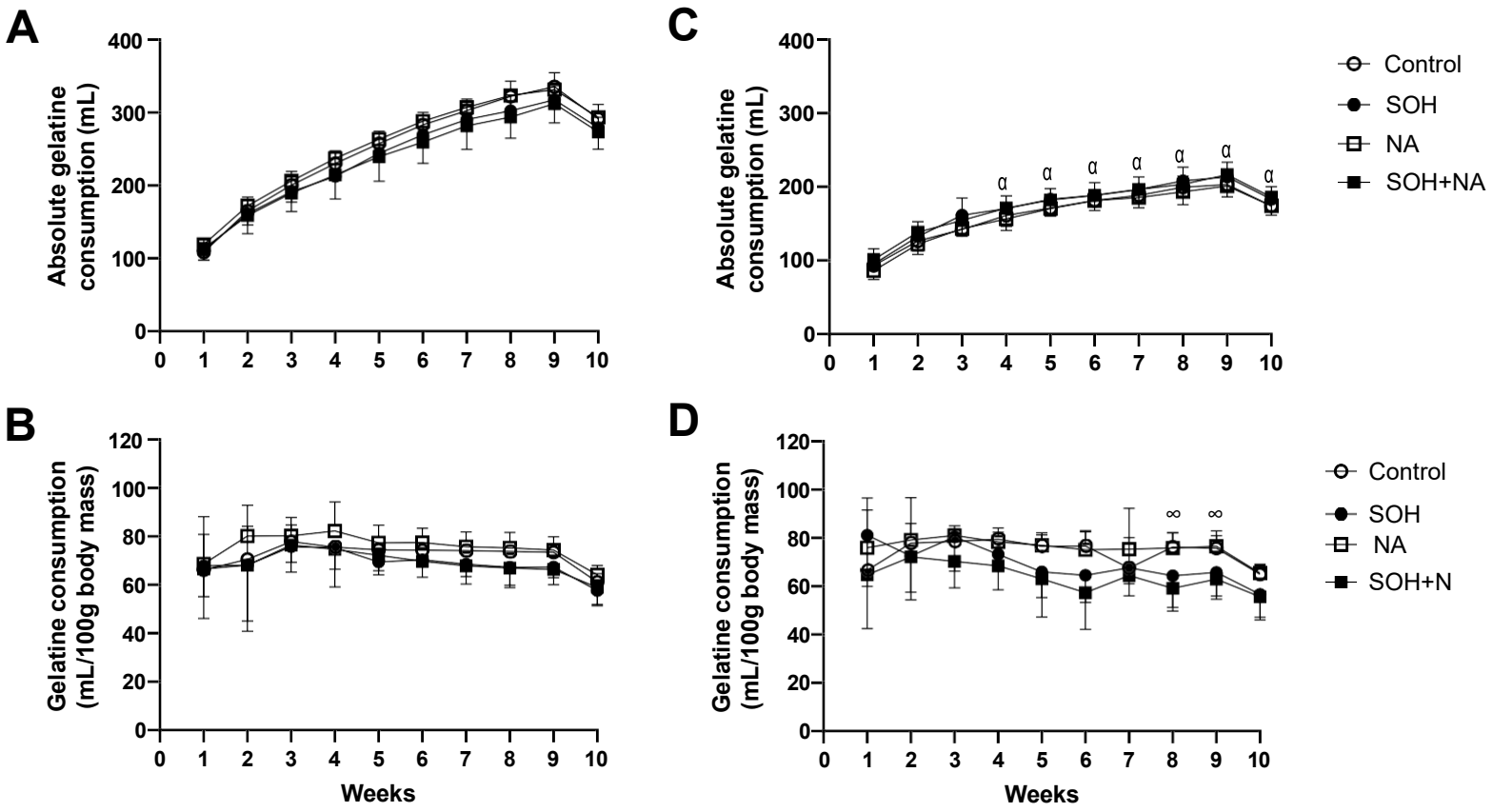
A
Absolute gelatine consumption (mL)
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
B
Gelatine consumption (mL/100g body mass)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Weeks
C
Absolute gelatine consumption (mL)
α
Control
SOH
NA
SOH+NA
D
Gelatine consumption (mL/100g body mass)
∞
Control
SOH
NA
SOH+N
Weeks

Figure 3.2: The weekly absolute (A and C) and relative (B and D) gelatine consumption in male (left panel) and female (right panel) Sprague-Dawley rats. Data presented as means ± SEM. ∞$p<0.05$ control and NA compared to SOH+NA. α$p<0.05$ compared to all male groups. SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin.

3.4.4 Echocardiography

3.4.4.1 Cardiac mass, cardiac geometry, and left ventricular systolic function

Table 3.2 shows the effects of sweetened alcohol and naringin on cardiac mass, cardiac geometry, and left ventricular systolic function in male and female Sprague–Dawley rats. The group and sex effects were significantly different for relative heart masses (group: $F = 21.26$, $p < 0.0001$; sex: $F = 138.72$, $p < 0.0001$) and absolute heart masses (group: $F = 6.54$, $p < 0.0001$; sex: $F = 34.03$, $p < 0.0001$). The group and sex effects were significantly different for relative LV masses (group: $F = 28.42$, $p < 0.0001$; sex: $F = 195.68$, $p < 0.0001$) and absolute LV masses (group: $F = 3.62$, $p < 0.0001$; sex: $F = 18.11$, $p < 0.0001$). Male heart and LV masses were heavier ($p < 0.0001$) compared to female heart and LV masses. However, female control, NA, and SOH + NA

groups had heavier ($p < 0.01$) heart mass indexed to body mass compared to male control and SOH groups. Female control, NA, and SOH + NA groups had heavier ($p < 0.01$) LV mass indexed to body mass compared to the male control group. The group and sex effects were significantly different for LV end-diastolic diameter (group: $F = 42.75$, $p < 0.0001$; sex: $F = 297.96$, $p < 0.0001$), LV end-diastolic posterior wall thickness (group: $F = 6.72$, $p < 0.0001$; sex: $F = 27.97$, $p < 0.0001$), LV end-systolic diameter (group: $F = 5.41$, $p < 0.0001$; sex: $F = 37.47$, $p < 0.0001$), LV end-systolic posterior wall thickness (group: $F = 8.90$, $p = 0.0001$; sex: $F = 62.04$, $p < 0.0001$), and relative wall thickness (group: $F = 5.71$, $p < 0.0001$; sex: $F = 17.17$, $p < 0.0001$) in male and female rats. Female SOH rats had greater LV end-diastolic posterior wall thickness, and relative wall thickness compared to female control rats ($p = 0.04$, and $p = 0.01$, respectively). Female SOH rats had greater relative wall thickness compared to female NA rats ($p = 0.01$). No significant differences in relative wall thickness were

observed between the female control, NA, and SOH + NA groups. LV end-diastolic diameter and LV end-systolic posterior wall thickness were greater ($p < 0.001$) in all males compared to all female groups. Female control and NA rats had reduced ($p < 0.01$) LV end-diastolic posterior wall thickness and LV end-systolic diameter compared to all male groups. Female SOH rats had greater ($p < 0.01$) relative wall thickness compared to all male groups. Female SOH + NA rats had greater ($p < 0.05$) relative wall thickness compared to male control and NA groups. The group and sex effects were significantly different for LV stroke volume (group: $F = 4.04$, $p = 0.0009$; sex: $F = 27.56$, $p < 0.0001$). There were no significant differences in group or sex effects for ejection fraction (group: $F = 0.49$, $p = 0.84$; sex: $F = 1.19$, $p = 0.28$). Although the sex effect was significantly different for fractional shortening ($F = 11.84$, $p = 0.0010$), no significant differences were observed for the group effect ($F = 1.83$, $p = 0.11$). There were no significant differences between groups in all markers of systolic function in male and female rats.

Table 3.2: Effects of sweetened alcohol and naringin on cardiac masses and geometry, and left ventricular systolic function in male and female Sprague-Dawley rats

	Males				Females			
	Control	SOH	NA	SOH+NA	Control	SOH	NA	SOH+NA
Cardiac masses and geometry								
Heart mass (g)	1.63 ± 0.06	1.55 ± 0.06	1.80 ± 0.06	1.64 ± 0.06	1.14 ± 0.02α	1.19 ± 0.10α	1.11 ± 0.04α	1.16± 0.05α
Heart mass/body mass x 103	3.52 ± 0.12∞	3.57 ± 0.13	3.52 ± 0.20∞	3.90 ± 0.12	4.41 ± 0.09	4.03 ± 0.09	4.32 ± 0.19	4.33 ± 0.12
LV mass (g)	0.68 ± 0.03	0.72 ± 0.03	0.72 ± 0.02	0.73 ± 0.02	0.47 ± 0.02α	0.48 ± 0.02α	0.49 ± 0.03α	0.50 ± 0.02α
LV mass/body mass x 103	1.48 ± 0.07∞	1.67 ± 0.07	1.59 ± 0.05	1.73 ± 0.04	1.83 ± 0.06	1.70 ± 0.06	1.89 ± 0.11	1.82 ± 0.08
LV end-diastolic diameter (mm)	7.50 ± 0.16	7.33 ± 0.16	7.46 ± 0.17	7.41 ± 0.16	5.46 ± 0.17α	5.29 ± 0.17α	5.43 ± 0.16α	5.32 ± 0.16α

LV end-diastolic posterior wall thickness (mm)	1.70 ± 0.07	1.78 ± 0.07	1.66 ± 0.07	1.72 ± 0.08	1.27 ± 0.08α	1.62 ± 0.08*#	1.31 ± 0.07α	1.56 ± 0.07
LV end-systolic diameter (mm)	3.86 ± 0.16	3.87 ± 0.16	3.88 ± 0.16	3.89 ± 0.17	3.11 ± 0.17α	3.19 ± 0.17	3.09 ± 0.16α	3.22 ± 0.16
LV end-systolic posterior wall thickness (mm)	3.12 ± 0.11	3.11 ± 0.11	3.06 ± 0.11	3.12 ± 0.11	2.51 ± 0.11α	2.48 ± 0.11α	2.51 ± 0.11α	2.49 ± 0.11α
Relative wall thickness	0.46 ± 0.02β	0.49 ± 0.02	0.45 ± 0.02β	0.47 ± 0.03	0.47 ± 0.03	0.62 ± 0.03*#α	0.49 ± 0.02	0.57 ± 0.02
Systolic function								
LV stroke volume (ml)	0.53 ± 0.03	0.50 ± 0.03	0.53 ± 0.03	0.51 ± 0.03	0.41 ± 0.03	0.40 ± 0.03	0.40 ± 0.03	0.40 ± 0.03
Ejection fraction (%)	80.31 ± 2.19	78.27 ± 2.19	78.74 ± 2.19	78.21 ± 2.31	81.99 ± 2.31	79.74 ± 2.31	82.04 ± 2.19	78.76 ± 2.19
Fractional shortening (%)	48.31 ± 2.51	46.94 ± 2.51	47.93 ± 2.51	47.29 ± 2.65	42.87 ± 2.65	39.74 ± 2.65	41.88 ± 2.51	41.02 ± 2.51

Data presented as mean ± SEM. *p<0.05 compared to control. #p<0.05 compared to NA. αp<0.05 compared to all male groups. βp<0.05 compared to female SOH+NA group. ∞p<0.05 compared to female control, NA and SOH+NA. LV: Left ventricle; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin.

3.4.4.2 Left ventricular diastolic function

3.4.4.2.1 Pulsed Doppler indices

Table 3.3 shows the effects of sweetened alcohol and naringin on pulsed Doppler indices (E and E/A) in male and female Sprague–Dawley rats. There were no significant differences observed in the group and sex effects for E velocity (group: F = 0.12, p = 0.99; sex: F = 0.00, p = 0.96) and E/A (group: F = 0.00, p = 1.00; sex: F = 0.01, p = 0.91) in male and female rats.

3.4.4.2.2 Tissue Doppler indices

Figure 3.3 shows the effects of sweetened alcohol and naringin on tissue Doppler indices in male and female Sprague–Dawley rats. The group and sex effects were significantly different for lateral e' (group: F = 15.41, p < 0.0001; sex: F = 52.18, p < 0.0001), e'/a' (group: F = 16.94, p < 0.0001; sex: F = 61.35, p < 0.0001), and E/e' (group: F = 14.25, p < 0.0001; sex: F = 34.55, p < 0.0001) in male and female rats. There were no significant differences in all tissue Doppler indices in male rats (Figure 3.3a–c). Female SOH (mean ± SEM: 3.11 ± 0.29 cm/s) and SOH + NA (mean ± SEM: 3.40 ± 0.28 cm/s) groups had reduced lateral e' compared to control (mean ± SEM: 5.33 ± 0.29 cm/s) and NA (mean ± SEM: 5.30 ± 0.28 cm/s) groups (p < 0.0001; Figure 3.3d). Female SOH (mean ± SEM: 0.64 ± 0.12) and SOH + NA (mean ± SEM: 0.75 ± 0.11) groups had reduced e'/a' compared to control (mean ± SEM: 1.57 ± 0.12) and NA (mean ± SEM: 1.51 ± 0.11) groups (All p < 0.0001; Figure 3.3e). Female SOH (mean ± SEM: 24.21 ± 1.37) and SOH + NA (mean ± SEM: 23.16 ± 1.31) groups had increased E/e' compared to control (mean ± SEM: 13.35 ± 1.37) and NA (mean ± SEM: 13.12 ± 1.31) groups (p < 0.0001; Figure 3.3f). No significant differences were observed between the female SOH and SOH + NA groups. All male groups had higher

e', e'/a' and reduced E/e' compared to female SOH and SOH + NA groups (All $p < 0.0001$)

Table 3.3: Effects of sweetened alcohol and naringin on left ventricular diastolic function (pulsed Doppler indices) in male and female Sprague-Dawley rats.

	Males				Females			
	Control	SOH	NA	SOH+NA	Control	SOH	NA	SOH+NA
E (cm/s)	70.75 ± 3.13	73.20 ± 3.13	70.70 ± 3.13	70.89 ± 3.30	70.00 ± 3.30	72.06 ± 3.30	70.60 ± 3.13	72.50 ± 3.13
E/A	1.67 ± 0.13	1.65 ± 0.13	1.66 ± 0.13	1.65 ± 0.14	1.68 ± 0.14	1.66 ± 0.14	1.66 ± 0.13	1.67 ± 0.13

Data presented as means ± SEM. LV: Left ventricle; E: early; A: late diastolic inflow velocities; E/A: ratio of early and late diastolic inflow velocities; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin.

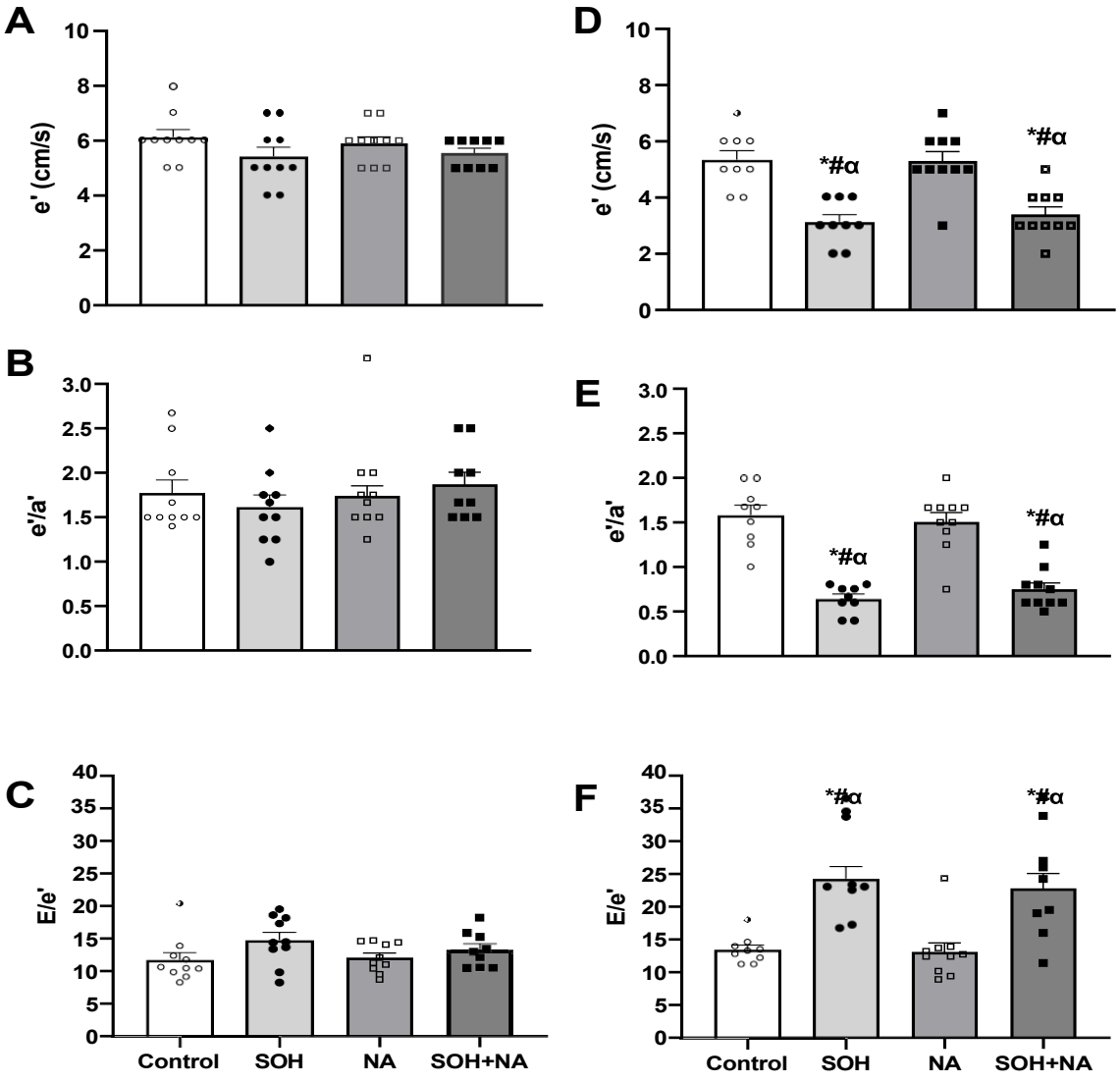

Figure 3.3: Effects of sweetened alcohol and naringin on lateral e' (A & D), e'/a' (B & E), and E/e' (C & F) in male (left panel) and female (right panel) Sprague-Dawley rats. Data presented as mean ± SEM. *p<0.0001 compared to control. #p<0.0001 compared to NA. αp<0.0001 compared to all male groups. LV: left ventricular; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin. e', peak velocity during early diastole; e'/a'; ratio of early to late peak velocities during diastole; E/e', ratio of early diastolic filling to peak velocity during early diastole.

3.4.5 Cardiomyocyte diameters

Figure 3.4 shows the effects of sweetened alcohol and naringin on LV cardiomyocyte diameters in longitudinal sections in male and female Spraque-Dawley rats. The group and sex effects were significantly different for the LV cardiomyocyte diameters (group: $F = 14.13$, $p < 0.0001$; sex: $F = 75.39$, $p < 0.0001$) in male and female rats. There were no statistical differences in LV cardiomyocyte diameters between the groups in male rats (Figure 3.4a, b). In females, LV cardiomyocyte diameters were significantly greater in the SOH (mean ± SEM: 17.79 ± 0.38 μm) compared to the control (mean ± SEM: 15.71 ± 0.38 μm; $p = 0.005$) and NA groups (mean ± SEM: 15.81 ± 0.38 μm; $p = 0.008$; Figure 3.4c, d). All male groups had greater LV cardiomyocyte diameters compared to the female control, NA, and SOH + NA groups ($p < 0.05$).

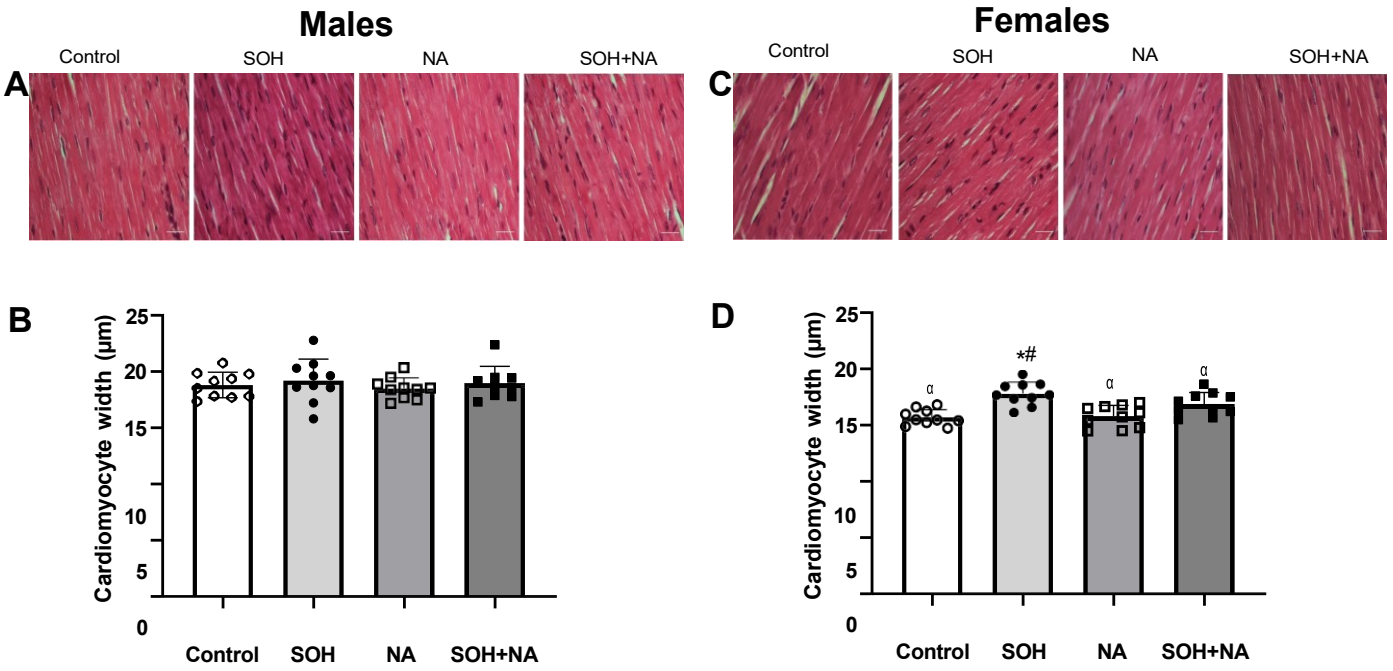

Figure 3.4: Effects of sweetened alcohol and naringin on LV cardiomyocyte diameters in longitudinal sections of male (left panel) and female (right panel) Sprague-Dawley rats. Representative H&E-stained micrographs imaged at x40 objective (x400 magnification) viewed in bright-field (A & C). LV cardiomyocyte diameters (B & D) measured from the H&E-stained sections. Data presented as means ± SEM. *p<0.05 compared to control. #p<0.05 compared to NA. αp<0.0001 compared to all male groups. LV: Left ventricle; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin.

3.4.6 Collagen area fraction

Figure 3.5 shows the effects of sweetened alcohol and naringin on LV collagen area fraction in male and female Sprague–Dawley rats. There were no visual differences in collagen accumulation between the groups of male rats when observed under bright filled (Figure 3.5A) or polarized light (Figure 3.5C). There was greater collagen accumulation in female SOH and SOH + NA rats compared to control and NA rats as evidenced by the increased red staining of cardiac tissue section visualized under bright-filled microscopy (Figure 3.5B) and large collagen fibers in orange or yellow visualized under polarized light (Figure 3.5D).

The group and sex effects were significantly different for the collagen area fraction (group: $F = 40.53$, $p < 0.0001$; sex: $F = 87.89$, $p < 0.0001$) in male and female rats. There were no significant differences in the collagen area fraction between the groups in male rats (Figure 3.5e). In females, the collagen area fraction was significantly greater in the SOH group (mean ± SEM: 9.12 ± 0.52%) compared to the control (mean ± SEM: 1.58 ± 0.52%; $p < 0.0001$), and NA (mean ± SEM: 2.15 ± 0.51%; $p < 0.0001$) groups (Figure 3.5f). In females, the collagen area fraction was significantly greater in the SOH + NA group (mean ± SEM: 8.71 ± 0.47%) compared to the control (mean ± SEM: 1.58 ± 0.52%; $p < 0.0001$) and NA (mean ± SEM: 2.15 ± 0.51%; $p < 0.0001$) groups (Figure 3.5f). No significant differences in collagen area fraction were observed between SOH and SOH + NA female groups ($p = 0.99$; Figure 3.5f). All male groups had reduced collagen area fraction compared to female SOH and SOH + NA groups (All $p < 0.0001$).

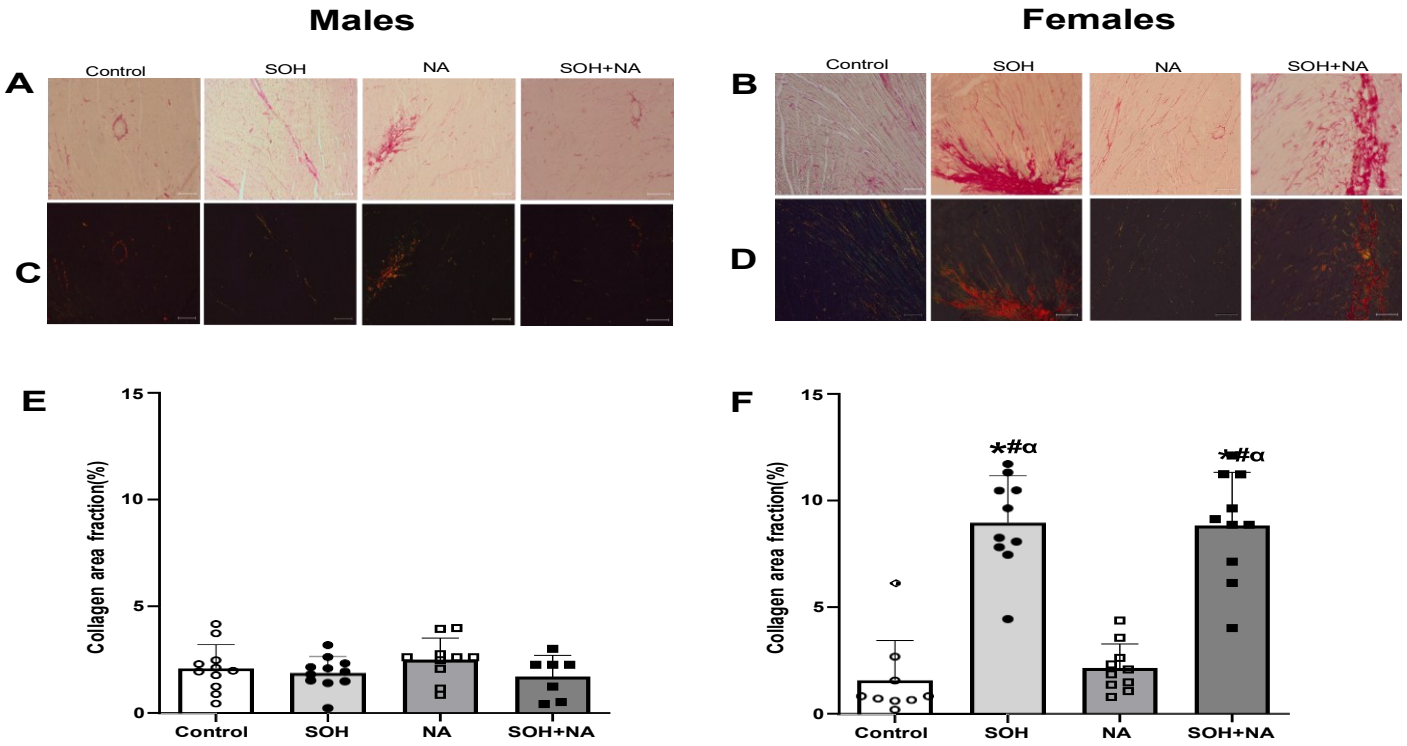

Figure 3.5: Effects of sweetened alcohol and naringin on collagen area fraction in LV tissue of male and female Sprague-Dawley rats. Representative Picrosirius red stained micrographs imaged at x10 objective (x100 magnification) viewed in bright-field (A & B) and under polarized light (C & D). Collagen area fraction (E & F) calculated from picrosirius red-stained sections. Data presented as means ± SEM. *p<0.05 compared to control. #p<0.05 compared to NA. αp<0.05 compared to all male groups. LV: Left ventricle; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin.

3.4.7 Associations between the collagen area fraction and tissue Doppler indices

Figure 3.6 shows the associations between the collagen area fraction and tissue Doppler indices. Reduced e' was associated with increased collagen area fraction (r = −0.35; p = 0.0018). Reduced e'/a' was associated with collagen area fraction (r = −0.31; p = 0.0069). Increased E/e' was associated with collagen area fraction (r = 0.33; p = 0.0039; Figure 3.6a). After adjusting for blood pressure, the results remained materially unaltered (e': partial r = −0.34 and p = 0.0033; e'/a': partial r = −0.30 and p = 0.0147; E/e': partial r = 0.31 and p = 0.0095; Figure 3.6b).

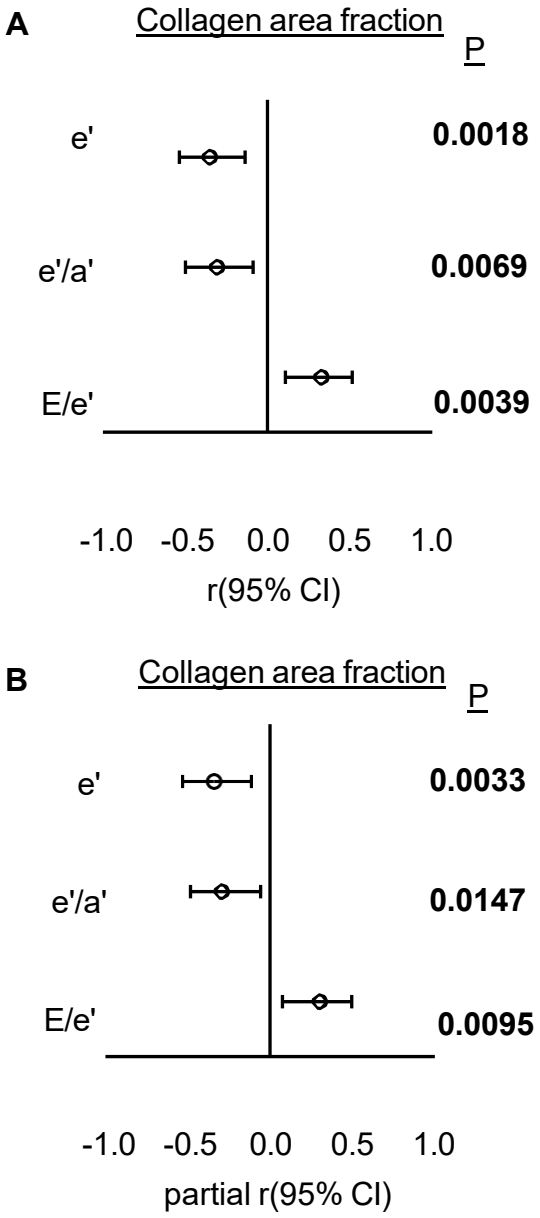

Figure 3.6: Associations between the collagen area fraction and measures of left ventricular diastolic function. A: before adjusting for BP, B: after adjusting for BP. e', peak velocity during early diastole; e'/a'; ratio of early to late peak velocities during diastole; E/e', ratio of early diastolic filling to peak velocity during early diastole. Open circles represent the correlation coefficient (r or partial r), and horizontal lines represent the 95% confidence intervals (CI).

3.5 Discussion

The current study investigated the effects of sweetened alcohol and naringin on cardiac function in male and female adolescent Sprague–Dawley rats. The main findings of this study were that sweetened alcohol and naringin did not affect gelatine consumption in male rats. However, in female rats, the combination of sweetened alcohol and naringin led to decreased gelatine consumption. In males, sweetened alcohol or naringin did not exert an impact on cardiac geometry, systolic function, or diastolic function. However, in females, the consumption of sweetened alcohol led to concentric remodeling without inducing alterations in LV mass and blood pressure. Although sweetened alcohol did not affect systolic function, it impaired LV relaxation and led to elevated LV filling pressures in females. Increased collagen area fraction was associated with impaired LV relaxation and elevated filling pressures. Naringin may have the potential to improve the sweetened alcohol-induced concentric remodeling, but not diastolic dysfunction in females. These findings highlight potential sex-specific differences in cardiac structure and function in response to exposure to sweetened alcohol and naringin.

3.5.1 Effects of sweetened alcohol and naringin on gelatine consumption

Relative gelatine consumption, which accounts for body weight, was not significantly different among the male groups. This indicates that when adjusted for body mass, the male rats consumed gelatine in similar proportions regardless of their experimental condition. This uniformity suggests that the interventions did not markedly affect the dietary behaviour of male rats in terms of gelatine consumption. In contrast, significant differences were noted in the relative gelatine consumption among female rats. Specifically, females in the SOH + NA group had reduced relative gelatine

consumption compared to both the control and NA groups in weeks 8 and 9 of the study; however, it was similar in the 10th week. It is unclear why this transient reduction in gelatine consumption in the SOH + NA group occurred. It does suggest that the combination of SOH and NA might influence the feeding behavior in female rats. Indeed, differences in preference for alcohol and fructose have previously been reported in rats (Li *et al*., 2019; Nieto & Kosten, 2017).

3.5.2 Effects of sweetened alcohol on cardiac geometry

In the current study, no differences were observed in markers of cardiac geometry in adolescent male rats. However, in adolescent females, the consumption of sweetened alcohol increased LV end-diastolic posterior wall thickness and relative wall thickness compared to controls without altering heart and LV masses. In addition, the consumption of sweetened alcohol marginally increased the LV cardiomyocyte diameters. These results suggest that the consumption of sweetened alcohol may have caused concentric remodeling in females. Indeed, in concentric remodeling, LV wall thickness and relative wall thickness increase before any detectable changes in LV mass. The individual effects of a high-fructose diet and alcohol on cardiac remodeling are controversial. While some studies have reported eccentric cardiac remodeling characterized by reduced LV wall thickness and relative wall thickness in rats subjected to fructose (Bouchard-Thomassin *et al*., 2011; Komnenov *et al*., 2020) or alcohol (Ai *et al*., 2020; Kim *et al*., 2003; Mouton *et al*., 2020), other studies have reported concentric cardiac remodeling and hypertrophy in rats exposed to fructose (Wang *et al*., 2023; Xing *et al*., 2010) or alcohol (Fernández-Solà & Porta, 2016). Interestingly, Wu *et al*. (2018) reported that the coadministration of fructose and alcohol (6% fructose + 4% ethanol) led to improvements in myocardial hypertrophy

compared to rats only consuming a high fructose diet (6% fructose) (Wu *et al*., 2018). The discrepancies in these findings may be due to specific variations in dietary conditions, age, as well as differences in rat strain and sex. Nevertheless, in the current study, we found that long-term consumption of sweetened alcohol may result in more pronounced LV mass changes and concentric hypertrophy in females. Although hypertension is a significant contributor to the cardiac remodeling process (Tomek & Bub, 2017), in the present study, blood pressures were similar between the control and SOH groups. This suggests that concentric remodeling in females occurred independently of changes in blood pressure. Indeed, cardiac remodeling can be induced not only by hypertension but also by inflammatory and fibrotic responses (Wang *et al*., 2023). In the current study, the consumption of sweetened alcohol increased LV total collagen content compared to the control group. Indeed, high fructose (Wang *et al*., 2023; Xing *et al*., 2010) or alcohol consumption (Lluís *et al*., 2011; Mouton *et al*., 2020) have been associated with cardiac fibrosis. In the heart, both fructose and alcohol may induce oxidative stress and inflammation, which then activate pathways that promote increased collagen formation and fibrosis (Kang *et al*., 2015; Zhang *et al*., 2016). Therefore, our findings suggest that the consumption of sweetened alcohol resulted in increased myocardial fibrosis, which could have led to concentric remodeling in females.

3.5.3 Effects of sweetened alcohol on diastolic function

In the current study, sweetened alcohol did not affect pulsed Doppler indices in male and female Sprague–Dawley rats. In agreement with our study, Wu *et al*. (2018) reported no changes in the E/A ratio in 8-week-old rats exposed to sweetened alcohol (6% fructose +4% ethanol) for 8 weeks (Wu *et al*., 2018). Despite no changes in pulsed Doppler indices, the consumption of sweetened alcohol in the current study led to

impaired tissue Doppler indices, including reduced lateral e' and e'/a' and increased E/e' in female rats. In addition, increased collagen area fraction was associated with impaired LV relaxation and elevated filling pressures. Although Wu *et al*. (2018) did not report on tissue Doppler indices, previous studies have reported impaired LV relaxation (reduced lateral e') and increased filling pressures (E/e') in rats fed a high fructose diet (Xing *et al*., 2010) and alcohol (Catena *et al*., 2016). In this regard, the consumption of sweetened alcohol may have impaired passive LV relaxation by promoting excessive collagen production and deposition that in turn, increased myocardial fibrosis. The increased myocardial fibrosis and impaired LV relaxation and stiffness caused by the sweetened alcohol may therefore lead to increased LV filling pressures (E/e'). In the current study, sweetened alcohol increased LV pressures despite the normal appearance of the E/A ratio. Indeed, increases in LV filling pressures drive a compensatory increase in the early diastolic filling (E), resulting in a pseudonormal filling pattern. Therefore, in the present study, the consumption of sweetened alcohol most likely caused moderate-to-severe diastolic dysfunction. The mechanisms by which fructose and alcohol impair LV relaxation and increase filling pressures are multifactorial. In addition to promoting fibrosis-mediated myocardial stiffness, fructose and alcohol may contribute to diastolic dysfunction by modifying calcium homeostasis, impacting contractile proteins, inducing oxidative stress, increasing lipogenesis, and promoting lipid accumulation in the myocardium (Kang *et al*., 2016; Laonigro *et al*., 2009; Li *et al*., 2022; Rasoul *et al*., 2023; Tsermpini *et al*., 2022; Wang *et al*., 2015; Zhang *et al*., 2016). Future studies should investigate these molecular mechanisms in more detail to gain a deeper understanding of their specific contributions.

3.5.4 Effects of sweetened alcohol on systolic function

In this study, the consumption of sweetened alcohol did not affect systolic function in both male and female rats. While some studies have reported that the individual consumption of fructose (Wang *et al*., 2023; Zhang *et al*., 2016) and alcohol (Ai *et al*., 2020; Hoek *et al*., 2022; Jung *et al*., 2003; Mouton *et al*., 2020) can adversely affect systolic function, characterized by impaired stroke volume, ejection fraction, and fractional shortening; others have not (Wang *et al*., 2023; Zhang *et al*., 2016). The majority of studies that have reported reduced ejection fraction and fractional shortening (Ai *et al*., 2020; Li *et al*., 2016; Wang *et al*., 2023; Zhang *et al*., 2016) have administered fructose and alcohol at high doses, and for a longer duration of intervention, which could partly explain the discrepancies in the results. Nonetheless, similar to our results, Wu *et al*. (2018) reported no changes in systolic function in rats receiving sweetened alcohol. The assessment of systolic function in clinical practice often relies on LV ejection fraction, a measure influenced by chamber size and pressure. As a result, it serves as a more reliable indicator for evaluating ventricular-arterial coupling than contractility (Borlaug *et al*., 2009). In our study, the use of more sensitive echocardiographic parameters, specifically Speckle Tracking Imaging (Dandel *et al*., 2009) may have facilitated the early detection of LV systolic dysfunction. In this context, research has indicated that myocardial deformation properties, specifically LV circumferential and longitudinal strain and strain rate may be compromised before any noticeable changes in systolic chamber function (Kucuk *et al*., 2017). Future studies should therefore use more sensitive measures of systolic function to explore the effect of sweetened alcohol on systolic performance.

3.5.5 Sex-specific effects of sweetened alcohol on cardiac remodeling and diastolic function

Estrogen possesses anti-inflammatory and vasodilatory properties, producing a cardioprotective environment for women (Iorga *et al*., 2017; Xiang *et al*., 2021). Nevertheless, our study showed that sweetened alcohol caused concentric remodeling and diastolic dysfunction in female rats, and not in their male counterparts. One plausible explanation for this discrepancy may be due to the interplay between sweetened alcohol and other physiological factors, specifically visceral adiposity. In the current study, female SOH rats had increased visceral adiposity indexed to body mass compared to male rats. Indeed, studies have reported that fructose consumption has a more pronounced effect on visceral adiposity in female rats compared to males (Kovačević *et al*., 2021). In addition, the accumulation of excess visceral adipose tissue plays an important role in the development of diastolic dysfunction and heart failure with a preserved ejection fraction in women more than in men (Sorimachi *et al*., 2021). Hypertrophic adipocytes, prevalent in visceral adiposity may secrete inflammatory markers such as tumor necrosis factor-alpha, interleukin 1β, interleukin 6, adipokines such as adiponectin, leptin, resistin, chemotactic protein 1 (MCP-1), and transforming growth factor (TGF)-β, (Farkhondeh *et al*., 2020) thereby promoting a state of chronic low-grade inflammation(Kolb, 2022). The increased circulating levels of these inflammatory markers may cause damage to the endothelium, leading to the upregulation of soluble intercellular adhesion molecules that increase the production of ROS (Paulus & Tschöpe, 2013). ROS decreases the bioavailability of nitric oxide and increases the production of peroxynitrites (Schulz *et al*., 2008). This, in turn reduces the production of cyclic guanosine monophosphate and lowers protein kinase G (PKG) activity within the myocardium (van Heerebeek *et al*., 2012). These altered signaling pathways and PKG activity result in increased cardiac remodeling, diastolic stiffness, and dysfunction through downstream hypophosphorylation of the protein, titin (Paulus & Tschöpe, 2013). This ultimately leads to impaired diastolic function and

concentric cardiac remodeling. Therefore, the sweetened alcohol-induced increased visceral adiposity may have triggered a cascade of inflammatory events leading to the remodeling process in females, thus increasing their susceptibility to diastolic dysfunction. These results shed light on the complexity of gender-specific responses to sweetened alcohol-induced cardiac effects and highlight the importance of gaining a better understanding of the underlying physiological mechanisms contributing to diastolic function impairment and cardiac remodeling in female rats. Further studies are crucial to elucidate the interactions between visceral adiposity, inflammation, and other contributing factors that collectively influence the observed gender-specific outcomes in response to sweetened alcohol administration.

3.5.6 Effects of naringin on sweetened alcohol-induced concentric remodeling and diastolic dysfunction

In the current study, naringin may have potentially improved the sweetened alcohol-induced concentric remodeling as the LV posterior wall thickness, relative wall thickness, and LV cardiomyocyte diameters in the SOH + NA group were similar to those of the controls. Previous studies have shown the potential therapeutic and cardioprotective effects of naringin in various experimental models of diet-induced cardiac injury, diabetic cardiomyopathy, and ischemic heart diseases (Malakul & Pengnet, 2018; Park *et al*., 2018; Pengnet *et al*., 2019; Viswanatha *et al*., 2022). Park *et al*. (2018) reported that naringin prevented the generation of ROS and mitochondrial dysfunction in cardiomyocytes exposed to fructose, which in turn decreased the hypertrophy of the cardiomyocytes (Park *et al*., 2018). Moreover, naringin prevented fructose-induced cardiomyocyte apoptosis by obstructing ROS-dependent ATM-mediated p53 signaling (Park *et al*., 2018). To the best of our knowledge, the cardioprotective effects of naringin have not been explored in alcoholic models.

Although naringin may have marginally improved the sweetened alcohol-induced concentric remodeling, it did not improve the severity of fibrosis since the total collagen content was similar in SOH + NA and SOH groups but was both significantly higher compared to controls. Therefore, it is plausible that naringin did not directly impact collagen formation, but rather it may have improved indices of concentric remodeling through alternative mechanisms, such as the reduction in ROS formation as reported in other studies (Akin *et al*., 2022; Bruno *et al*., 2022; Laonigro *et al*., 2009). Future studies should consider these mechanisms to further explore the potential of naringin in the improvement of sweetened alcohol-induced concentric remodeling at a molecular level. Although naringin may have marginally improved concentric remodeling, it did not ameliorate the sweetened alcohol-induced diastolic dysfunction. Concentric remodeling has the potential to contribute to diastolic dysfunction, as the increased stiffness of the heart muscle may affect its ability to relax during diastole (Røe *et al*., 2017). Given the observed improvement in sweetened alcohol-induced concentric remodeling with naringin, a longer duration of treatment and higher doses of naringin may have eventually led to improvements in diastolic function.

Therefore, these factors should be considered in future studies. Additionally, understanding the broader metabolic responses that lead to reduced gelatine consumption in females could provide insights into the interconnected nature of diet and cardiac health in the presence of such substances. The present study has further limitations. While previous studies have extensively examined the individual effects of fructose (Bouchard-Thomassin *et al*., 2011; Komnenov *et al*., 2020; Wang *et al*., 2023; Xing *et al*., 2010; Zhang *et al*., 2016) and alcohol (Ai *et al*., 2020; Fernández-Solà & Porta, 2016; Kim *et al*., 2003; Mouton *et al*., 2020) on cardiac function, these studies did not employ gelatine as a vehicle. Future investigations would benefit from the

incorporation of 20% fructose and 10% ethanol solely within gelatine to provide a clearer comparison between individual and combined effects. In addition, the use of a single dose or concentration for each compound of interest (fructose at 20%, alcohol at 10%, and naringin at 50 mg/kg) may have contributed to the absence of some of the observable effects. Future investigations need to explore a wider range of doses to elucidate potential dose–response relationships more comprehensively. As Gomori's trichrome and wheat germ agglutinin (WGA) stains offer better specificity and quantitative accuracy for measuring cardiomyocyte size compared to the more general H&E staining, further investigation in this regard may provide additional insights in the assessment of cardiac remodeling. Finally, the lack of measurement of blood alcohol concentration following consumption of ethanol-containing gelatine is an aspect that warrants further investigation in future studies since insights into how gelatine matrices influence alcohol release and absorption can inform alcohol metabolism considerations and health implications.

3.6 Conclusion

In males, neither sweetened alcohol nor naringin had any impact on cardiac geometry or diastolic function. Conversely, in females, the consumption of sweetened alcohol resulted in increased visceral obesity and concentric remodeling without inducing alterations in LV mass and blood pressure. Sweetened alcohol-impaired LV relaxation and elevated LV filling pressures in females. Notably, naringin improved the sweetened alcohol-induced concentric remodeling but did not mitigate the diastolic dysfunction and visceral obesity induced by sweetened alcohol in females. These findings contribute to a better understanding of the effects of sweetened alcohol and naringin on cardiac health and underscore the need for further research to unravel the underlying mechanisms that account for gender-specific differences.

Cardiovascular dysfunction has been associated with the development of metabolic dysfunction-associated fatty liver disease (Peng *et al*., 2022). The next chapter therefore, will look at the effects of sweetened alcohol and naringin on hepatic steatosis, trigleceride, fasting plasma glucose and insulin.

CHAPTER FOUR - EFFECTS OF NARINGIN ON HEPATIC STEATOSIS, AND OTHER METABOLIC PARAMETERS (FASTING BLOOD GLUCOSE, TRIGLYCERIDE AND INSULIN) IN MALE AND FEMALE SPRAGUE-DAWLEY RATS FED WITH SWEETENED ALCOHOL

4.1 Introduction

Sweetened alcohol is among the most widely consumed alcoholic beverages globally, particularly among adolescents and young adults (Fortunato *et al*., 2014). The beverage's sweet taste increases the palatability of the alcohol, resulting in a high amount of alcohol being consumed within a short time (Crews, 2015), especially by females (Siegel *et al*., 2016). This potentially increases the risk for the development of an array of metabolic disorders. Fructose is frequently used as a sweetener in the production of sweetened alcoholic beverages (Wakabayashi *et al*., 2021). Unfortunately, there is an increase in the prevalence of metabolic ailments, including fatty liver disease and obesity, due to high alcohol and fructose consumption (Herman & Birnbaum, 2021; Mitra *et al*., 2020).

Fatty liver disease (hepatic steatosis) is caused by an imbalance in lipid metabolism resulting from alcohol and/or fructose excess (Yan *et al*., 2021). Alcohol dysregulates several components of hepatic lipid metabolism, such as enhancing neutral lipid storage, fatty acid oxidation impairment, boosting *de novo* lipid synthesis, and increasing alcohol-induced hepatic fatty acid absorption (Jeon & Carr, 2020). Alcohol can also prevent the formation of very low-density lipoproteins (VLDL) and apolipoprotein B (ApoB), hindering the ability of the liver to release lipids smoothly (Tomita *et al*., 2004).

Furthermore, alcohol can change the distribution of body fat, with hepatic and abdominal fat accumulating more than peripheral fat (Suter, 2005). Alcohol has a 7.1 kcal/g caloric value, which is comparatively high in energy compared to carbohydrate and protein (with 4kcal/g) and boosts appetite, which raises caloric intake and creates a favourable energy balance (Suter, 2005). Consequently, alcohol consumption has been positively associated with visceral adiposity (Kim *et al*., 2012).

Similar to alcohol, fructose has detrimental effects in the body, particularly in the liver, due to its activation of various pro-inflammatory, fibrogenic, and carcinogenic signalling pathways (Muriel *et al*., 2021). Consuming fructose has been linked to hepatic steatosis induction via oxidative stress, elevated fatty acid synthesis, and the development of insulin resistance (Old *et al*., 2021). While numerous studies have documented findings on the individual effects of alcohol and fructose on metabolic conditions, including hepatic steatosis (Arafa *et al*., 2019; Wei *et al*., 2023), the combined effects of fructose and alcohol in the form of sweetened alcohol need further investigation to find strategic approaches to prevent and treat their negative metabolic effects.

Management of metabolic diseases, especially liver pathology, is associated with challenges worldwide, particularly in developing countries. The challenges range from financial constraints to the need for prescription of several drug combinations, each with associated adverse effects that hinder compliance, lack of access to healthcare facilities, and the unavailability of medications such as antihypertensives for blood pressure regulation, antidiabetic drugs for diabetes mellitus, lipid-lowering agents such as statins to manage dyslipidaemia (Saad *et al*., 2017).

Phytochemicals derived from medicinal plants are becoming increasingly popular in the treatment and prevention of an array of disease conditions, possibly due to their (plant-based medications) availability, affordability, and perceived holistic medicinal benefits (Bharti *et al.,* 2014). Naringin is one such phytochemical of interest.

Naringin, a flavonoid, is a major component of grapefruit, several other citrus fruits, and tomatoes (Bharti *et al.,* 2014). Notably, it has been associated with numerous biological processes, such as hepatoprotective, anti-inflammatory, anti-lipidemic, and

antioxidant effects (Jutrić *et al.,* 2022; Shilpa *et al.,* 2023; Zhou *et al.,* 2019). Flavonoids, including naringin and its metabolites, have been reported to be inhibitors of the hepatic enzyme Cytochrome P450 3A4 (CYP3A4), which metabolises many drugs and medications in the liver (Guttman & Kerem, 2022; Kondža *et al*., 2024). Although naringin can weakly inhibit CYP3A4 (Kondža *et al.,* 2024), alcohol may increase its activity (Feierman *et al*., 2003). Hence, they could balance each other out when taken together.

Naringin has been the subject of many studies and has shown potential for the management and prevention of liver and metabolic disorders linked to individual effects of alcohol and fructose (Kannappan *et al.,* 2010; Zhou *et al.,* 2019). However, based on the available literature, no study has explored the influence of naringin on hepatic steatosis and visceral obesity induced by the ingestion of sweetened alcohol. Interestingly, despite known sexual dimorphism in metabolic disease presentation, studies tend to focus on males while excluding females. Therefore, this study additionally intended to determine the effects of naringin and voluntary sweetened alcohol consumption on hepatic steatosis and visceral obesity in male and female Sprague-Dawley rats.

4.2 Methods

4.2.1 Study settings, study design, animal housing, and ethical approval

As described in Chapter Three - 3.3.1, 3.3.2 & 3.3.3

Table 4.1 shows the composition of the standard rat chow (LabChef Rodent Breeder, Nutritional Hub (Pty) Ltd, Stellenbosch 7602, South Africa) used in the study

Table4.1: Nutritional composition of standard rat chow

Ingredients	Composition
Energy content	2.8kcal
Protein	220g/kg
Moisture	10g/kg
Oils and fats	50g/kg
Linoleic acid	12g/kg
Fibre	40g/kg
Ash	70g/kg
Calcium	12g/kg

4.2.2 Body mass measurements

As described in Chapter Three – 3.3.4

4.2.3 Terminal procedures

As described in Chapter Three – 3.3.7

Blood was taken after a pinprick on the tail, and fasting glucose was measured using a calibrated glucometer (Contour plus Blood glucose monitoring system, Bayer (Pty) Ltd., Praha, Czech Republic).

4.2.4 Liver mass

Following termination, from each rat, the liver was removed, its weight determined, and a section of the accessory lobe was then preserved in 10% phosphate-buffered formalin for later processing for histological examination.

The liver and fat masses were determined on a Presica 310M digital balance (Precision Instruments, Johannesburg, South Africa). For each rat, the visceral fat mass relative to body mass was calculated by dividing the visceral fat mass by the terminal body mass and then multiplying by 100. The hepatosomatic index (hepatic mass relative to body weight) was similarly computed.

4.2.5 Determination of Serum Triglyceride, Insulin, and Fasting Blood Glucose Concentrations

Following a thoracotomy as described in chapter 3, section 3.3.7, a cardiac puncture was performed to obtain blood samples using a 10 mL syringe with a 21G hypodermic needle attached and transferred into 6 mL serum clot activator tubes. Serum was collected after the blood was centrifuged (DLAB, DM0412, USA) at 3000 x g for ten minutes. The serum was aliquoted at 0.25 ml into microtubes (Eppendorf, Hamburg, Germany), stored at -80°C, for insulin and TG assays.

A colorimetric kit [Elabscience ® Triglyceride (TG) Colorimetric Assay kit (single reagent, GPO-PAP method), Cat. No.: E-BC-K261-M. Houston, TX, USA] was used to determine the serum TG concentration. (sensitivity, 0.14mmol/L; detection range, 0.14-10 mmol/L, standard curve range, 0-12 mmol/L). For full protocol and procedures for TG determination using ELISA, see appendix 5B.

An enzyme-linked immunosorbent assay (ELISA) kit [Elabscience ® Rat INS (Insulin) ELISA kit, Cat. No.: E-EL-R3034. Houston, TX, USA] was used to determine the serum insulin concentration (sensitivity, 37.5 pg/ml; detection range, 62.5-400 pg/ml; standard curve range, 1-1000 pg/ml). For the full protocol and procedure for Insulin determination using ELISA, see appendix 5A.

4.2.6 Histological assessment of the liver

An automated tissue processor (Microm STP 120, ThermoScientific, MA, USA), was used to process the liver samples that had been stored in 10% phosphate-buffered formalin. Thereafter, the samples were embedded in paraffin wax, sectioned at 4 µm and then stained with haematoxylin and eosin (H&E) for steatosis assessment (see page 78, item 3.3.9 for a detailed H&E procedure). The slides were viewed at x40 objective (X100 magnification) under a light microscope [Axioskop 2 microscope; (Leica Biosystems, USA)] with a mounted Axiocam HRc2 camera, which uses ZEISS ZEN microscope software for image capture, and scored for steatosis. The H&E images were used to score for microvesicular and macrovesicular steatosis as described by Kleiner *et al* (Kleiner *et al*., 2005). The following criteria were used to grade the overall area impacted by each camera field at 40x objective (x100 magnification):

i. Less than 5% of fat droplets in the field were identified as grade 0 steatosis,

ii. 5–33% area occupied by fat droplets was identified as grade 1 steatosis,

iii. 34–66% area occupied was identified as grade 2 steatosis,

iv. A greater than 66% area occupied by fat droplets was identified as grade 3 steatosis.

The scoring was performed thrice for each slide, and the average was taken. Scoring was performed with the scorer blinded to the groups.

4.2.7 Statistical analysis

SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used to analyse the data, while GraphPad Prism version 9.4 (GraphPad Software Inc., San Diego,

USA) was used to create the graphs. For normalcy tests, Shapiro-Wilk tests were employed. Data are presented as mean ± standard deviation (SD) for normally distributed data and as median ± interquartile range (IQR) for skewed data. Two-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used to analyse group and sex differences in FBG, TG, serum insulin, liver, and adipose tissue masses. Repeated measures ANOVA was used to determine baseline and terminal body weights, as well as weekly food and water intake. The Kruskal–Wallis test followed by Dunn's *post hoc* test was used to analyse multiple-group data for steatosis grades. Statistical significance was set at a p-value of <0.05.

4.3 Results

4.3.1 Food and water intake

Figures 4.1 and 4.2 show the effects of naringin and sweetened alcohol on weekly food and water intake from week 1 to week 10 in male and female Sprague-Dawley rats. For the food intake, in male rats (Figure 4.2A), no difference ($p>0.05$) was observed in the first and second weeks of intervention between all the groups (SOH, NA and SOH+NA) compared to control. From the third to the tenth week, compared with control and NA groups, rats in SOH and SOH+NA groups had significantly decreased food intake ($p<0.05$). In female rats (Figure 4.2B), there were no significant differences in food intake between the groups from week 1 to week 8 ($p>0.05$). At week 9, compared with control, rats in the SOH group had significantly reduced food intake ($p<0.05$), and at the tenth week, compared with control and NA, rats in the SOH and SOH+NA groups had significantly decreased food intake ($p<0.05$).

The groups' water intake did not differ significantly in both male (Figure 4.3A) and female (Figure 4.3B) rats from week 1 to week 10 ($p>0.05$). Thus, in summary, sweetened

alcohol consumption led to decreased food intake that manifested earlier in males than in females. Naringin did not influence food and water intake.

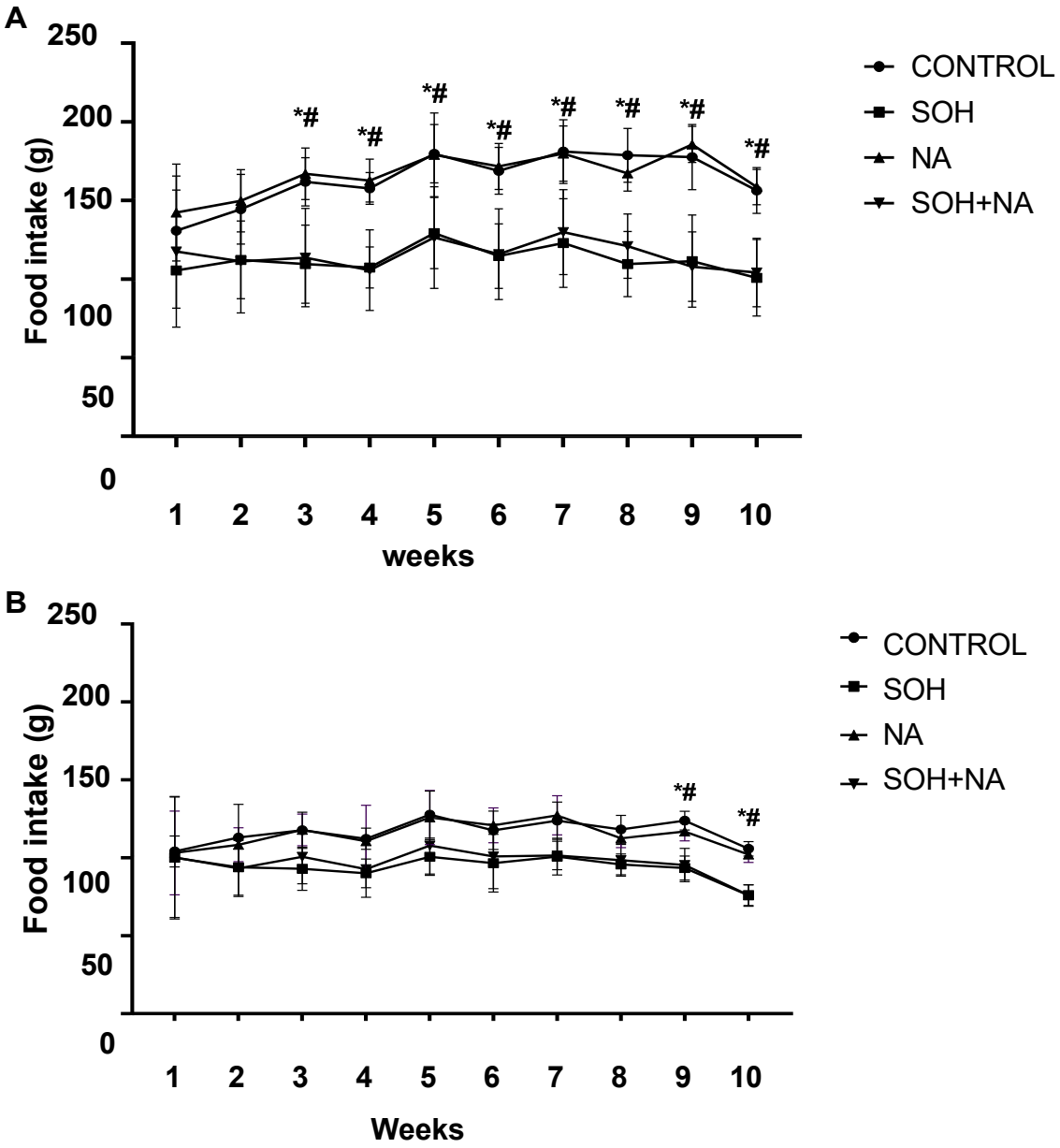

Figure 4.1: Effects of sweetened alcohol and naringin on weekly food intake in male. *p<0.05 compared to control; #p<0.05 compared to NA. Control: 0.5% fructose in 6% gelatine at 10ml/100g for 10 weeks (n= 20, 10 males, 10 females); SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n= 20, 10 males, 10 females). SOH: Sweetened

alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin. Data presented as means ± SD.

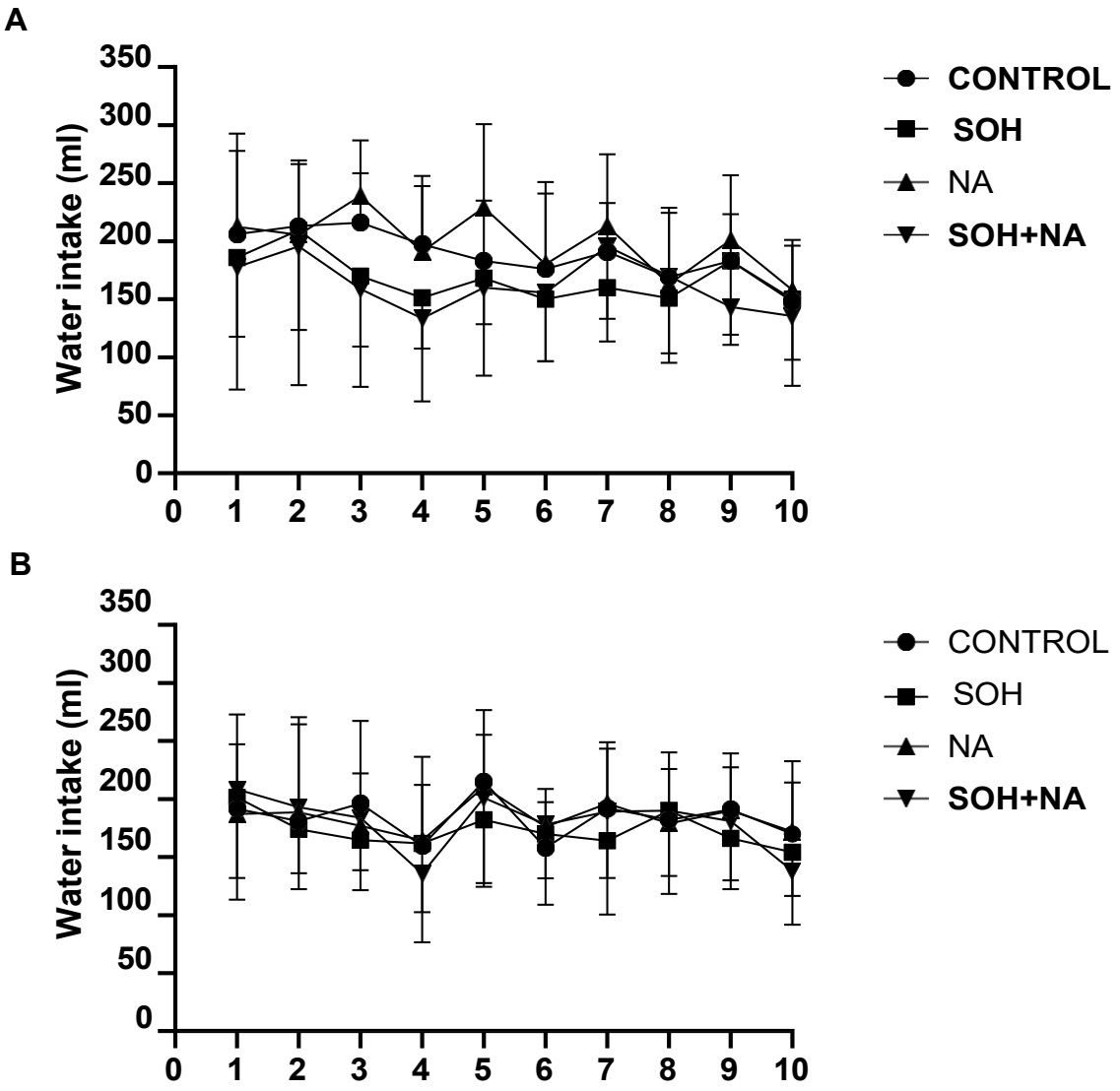

Figure 4.2: Effects of sweetened alcohol and naringin on weekly water intake in male: 0.5% fructose in 6% gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females); SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin. Data presented as means ± SD.

4.3.2 Liver masses

Table 4.2 shows the effects of naringin and sweetened alcohol on absolute and relative liver masses in male and female Sprague-Dawley rats. There were significant differences in group and sex effects for liver mass (group: $F=165.92$, $p<0.0001$; sex: $F=1147.91$, $p<0.0001$). There was no significant difference in the absolute liver mass across the groups ($p>0.05$). However, when liver mass was indexed to body mass, there were significant differences in group and sex effects for relative liver mass (group: $F=7.27$, $p<0.0001$; sex: $F=29.18$, $p<0.0001$). Specifically, compared to the control, rats in SOH+NA had increased relative liver mass in male and female rats ($p=0.01$, $p=0.04$, respectively). Additionally, compared with females, male rats had significantly ($p<0.0001$) higher absolute liver mass in all the groups.

Table 4.2: Effect of sweetened alcohol and naringin on the mass of the liver (absolute and relative) in male and female Sprague-Dawley rats.

	Sex	Control	SOH	NA	SOH+NA
Liver mass (g)	Males	12.24 ± 0.90$^{\Omega}$	11.63 ± 0.52$^{\Omega}$	12.21 ± 0.45$^{\Omega}$	11.81 ± 0.72$^{\Omega}$
	Females	7.25 ± 0.48	7.75 ± 0.56	7.26 ± 0.33	7.65 ± 0.55
Liver mass (%BM)	Males	2.62 ± 0.13	2.69 ± 0.87	2.68 ± 0.10	2.80 ± 0.10*
	Females	2.74 ± 0.61	2.85 ± 0.11	2.83 ± 0.10	2.90 ± 0.18*

*$p<0.05$ compared to control. $^{\Omega}p<0.0001$ compared to female rats. Control: 0.5% fructose in 6% gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females); SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). BM: Body Mass; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin. Data presented as means ± SD.

4.3.3 Serum triglyceride concentration, fasting blood glucose, and serum insulin

Table 4.3 shows the effects of naringin and sweetened alcohol on circulating metabolic parameters (TG, FBG, and insulin) in male and female Sprague-Dawley rats. There were no differences in group effect for TG ($F = 1.23$, $p=0.2976$), but there was a significant difference in sex effect for TG (sex: $F= 4.05$, $p=0.04$). For FBG, there were significant differences in group and sex effects (group: $F=28.65$, $p<0.0001$; sex: $F=6.28$, $p<0.0001$). For insulin, there was a significant difference in group effect ($F = 6.12$, $P<0.0001$), but no difference in sex effect ($F=2.86$, $p=0.09$). There was no significant difference in serum TG and FBG concentration across the groups in male rats ($p>0.05$). In female rats, compared to controls, rats in SOH+NA had reduced

serum TG concentration ($p=0.004$). Also, in female rats, compared to controls, and SOH, SOH+NA rats had increased FBG ($p=0.02$; $p=0.006$). Compared with all the groups, male rats in SOH+NA had significantly increased fasting serum insulin concentration ($p<0.05$). In female rats, compared to controls, rats in all the groups had decreased serum insulin concentration ($p<0.05$). Compared to male rats in all the groups, female rats in SOH+NA had significantly decreased serum TG concentration and increased ($p<0.05$) FBG concentration ($p<0.05$). In addition, compared to female control, male rats in the control and the SOH groups had decreased ($p<0.001$, $p=0.02$, respectively) serum insulin concentration, and compared to female SOH+NA, male rats in SOH+NA had significantly increased ($p=0.04$) serum insulin concentration.

Table 4.3: Effects of sweetened alcohol and naringin on serum triglyceride, fasting blood glucose and fasting serum insulin in male and female Sprague-Dawley rats.

Parameter	Sex	Control	SOH	NA	SOH+NA
Triglyceride (mmol/L)	**Males**	0.96 ± 0.15	1.00 ± 0.21	0.94 ± 0.15	1.01 ± 0.21
	Females	0.83 ± 0.32	0.55 ± 0.29	0.74 ± 0.34	0.47 ± 0.11*$^{\delta}$
Fasting blood glucose (mmol/L)	**Males**	3.33 ± 0.36	3.24 ± 0.38	3.32 ± 0.21	3.16 ± 0.31
	Females	3.50 ± 0.27	3.46 ± 0.25$^{\beta}$	3.68 ± 0.17	3.91 ± 0.42*$^{\delta}$
Fasting serum insulin (µU/mL)	**Males**	6.29.0 ± 2.18^{a}	7.53.0 ± 1.30$^{\beta a}$	7.31 ± 1.32	9.94 ± 1.20$^{*\#b}$
	Females	9.97 ± 1.82	7.78 ± 1.54*	7.87 ± 1.10*	7.63 ± 1.56*

*p<0.05 compared to control. $^{\#}$p<0.05 compared to NA. $^{\beta}$p<0.05 compared to SOH+NA. $^{\delta}$p<0.05 compared to all males. ^{a}p<0.05 compared to the female control. ^{b}p<0.05 compared to female SOH+NA. Control: 0.5% fructose in 6% gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 19, 9 males, 10 females. BM: Body mass; SOH: sweetened alcohol; NA: naringin; SOH+NA: sweetened alcohol + naringin. Data presented as means ± SD.

4.3.4 Liver histomorphometry

Figures 4.3 and 4.4 show representative photomicrographs of liver histological sections from male and female Sprague-Dawley rats, respectively. Fat accumulation, presenting as microvesicular steatosis (white arrows) and macrovesicular steatosis (black arrows) was more evident in the SOH group rats compared to control, NA, and SOH+NA groups in females. However, only microvascular steatosis was more evident in the SOH group rats compared to other groups in male rats. Quantitatively, there were significant differences in group and sex effects for macrovesicular steatosis (group: $F=16.11$, $p<0.0001$; sex: 34.34, $p<0.0001$), and microvesicular steatosis (group: $F = 6.05$, $p<0.0001$; sex: $F = 9.48$, $p<0.003$). The microvesicular steatosis scores were greater in SOH rats compared to other groups in male and female rats ($p<0.05$). Macrovesicular steatosis scores were also greater ($p<0.05$) in the SOH group in female rats compared to other groups, while in male rats, macrovesicular steatosis scores showed no statistically significant difference among all the groups ($p>0.05$) (Figure 4.3A). Female SOH rats had significantly increased ($p<0.0001$) macrovesicular steatosis scores [median (IQR) = 2 (2, 2)] compared to male SOH [median (IQR) = 0 (0, 0.7)]. However, there was no significant difference in micro-vesicular steatosis scores between male SOH [median (IQR) = 1 (0.25, 2)] and female SOH [median (IQR) = 2 (1.5, 3)] rats ($p>0.05$). In summary, sweetened alcohol caused microvesicular and macrovesicular steatosis in both male and female rats, with macrovesicular steatosis being more prominent in female rats. Naringin supplementation prevented the development of hepatic steatosis.

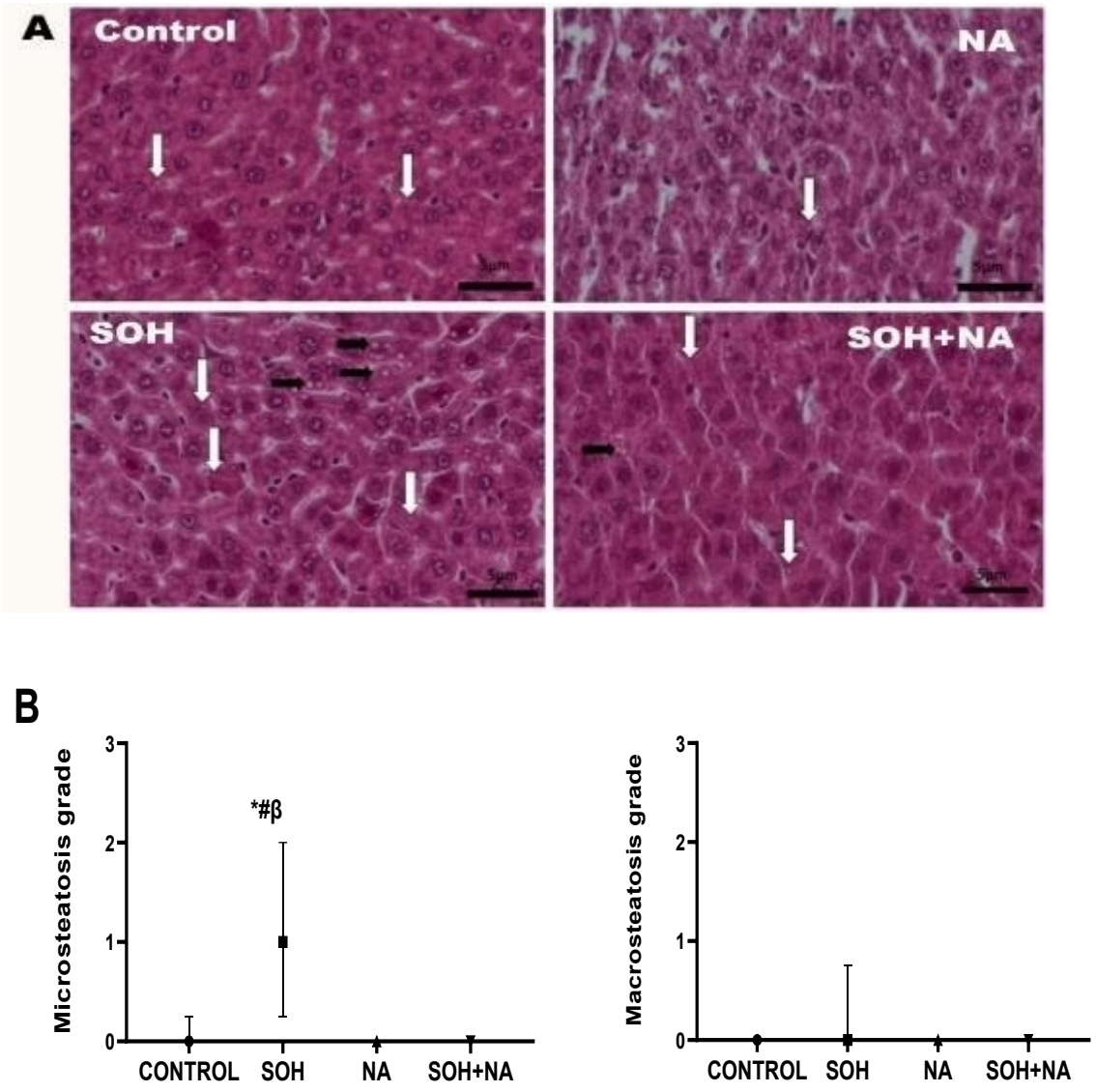

Figure 4.3: Effects of sweetened alcohol and naringin on hepatic steatosis in male Sprague-Dawley rats. Representative photomicrographs of liver histology (A) and steatosis score (B) from each treatment group (stained with H&E) imaged at x40 objective (x100 magnification). Microvesicular steatosis (White arrows), macrovesicular steatosis (Black arrows). Data presented as median ± IQR. *p<0.05 compared to control. #p<0.05 compared to NA. βp<0.05 compared to SOH+NA. SOH: sweetened alcohol; NA: naringin; SOH+NA: sweetened alcohol + naringin; H&E: Haematoxylin and Eosin. Scale bar = 5μm.

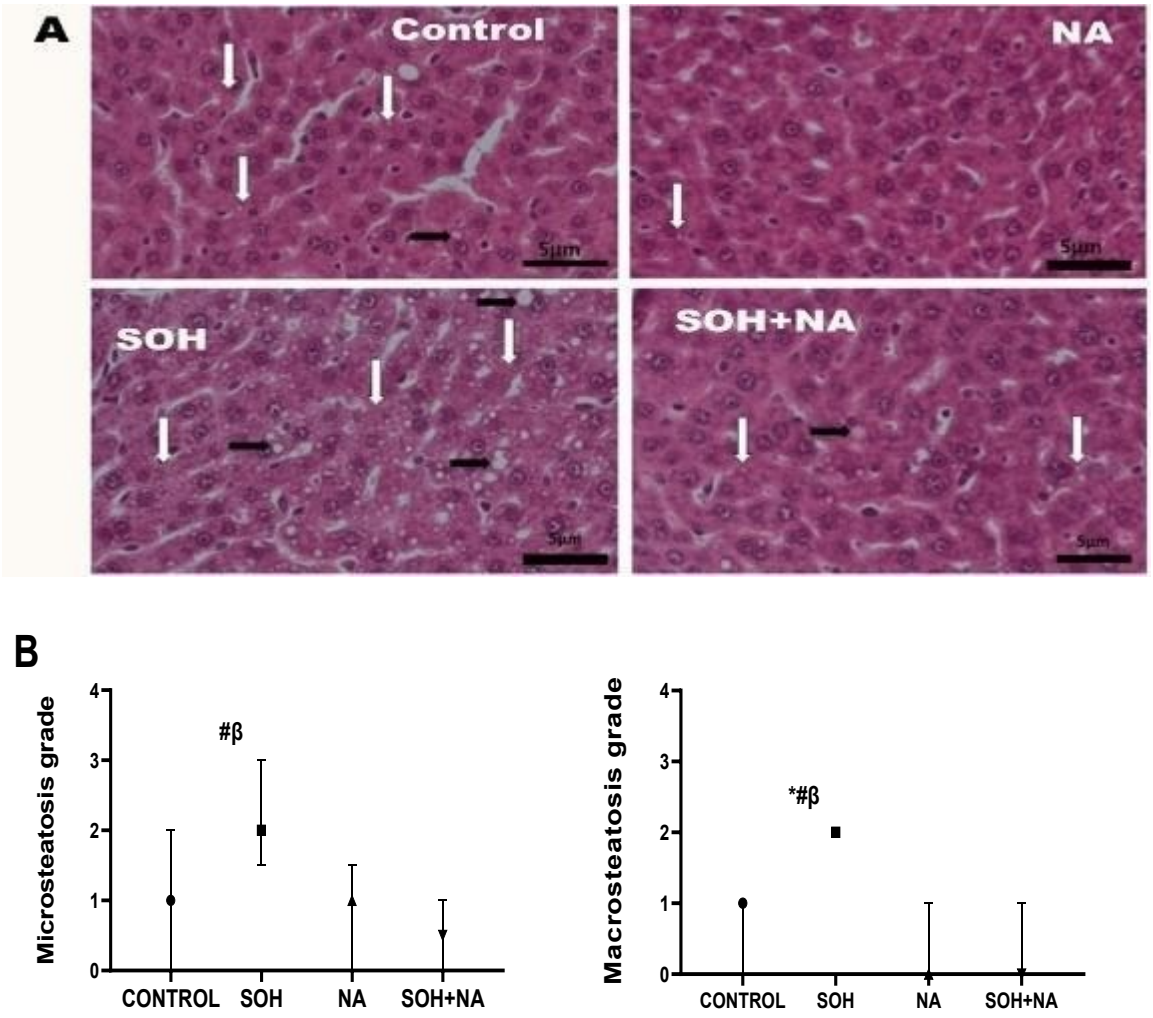

Figure 4.4: Effects of sweetened alcohol and naringin on hepatic steatosis in female Sprague-Dawley rats. Representative photomicrographs of liver histology (A) and steatosis score (B) from each treatment group (stained with H&E) imaged at x40 objective (x100 magnification). Microvesicular steatosis (White arrows), macrovesicular steatosis (Black arrows). Data presented as median ± IQR. *P<0.05 compared to control; p<0.05 compared to NA; βp<0.05 compared to SOH+NA. SOH: sweetened alcohol; NA: naringin; SOH+NA: sweetened alcohol + naringin; H&E: Haematoxylin and Eosin. Scale bar = 5µm.

4.4 Discussion

In the present study, I investigated the effects of naringin and sweetened alcohol on the metabolic response in male and female Sprague-Dawley rats. The major findings from this study are that sweetened alcohol led to decreased food intake in male and female rats, with the effects appearing earlier in males than in females. However, there was no effect on water intake or liver mass. Naringin supplementation alone did not affect liver mass, food, or water intake. However, naringin in combination with sweetened alcohol led to increased relative mass of the liver in both male and female rats. Sweetened alcohol had no effects on circulating TG, insulin, or FBG in both male and female rats; however, in female rats, compared to control, naringin supplementation in combination with sweetened alcohol led to decreased TG concentration and increased FBG concentration. Compared to all the groups, naringin supplementation with sweetened alcohol led to increased serum insulin concentration in male rats, and in female rats, sweetened alcohol alone, naringin supplementation alone, and in combination with sweetened alcohol led to decreased serum insulin concentration. Sweetened alcohol caused increased fatty infiltration (hepatic steatosis) in both male and female rats, with macro-vesicular steatosis more prominent in female rats. Naringin supplementation prevented the sweetened alcohol-induced hepatic steatosis.

4.4.1 Effects of naringin and sweetened alcohol on food and water intake

In this study, I showed that consumption of sweetened alcohol caused decreased food consumption with no effects on water intake. Naringin had no effects on food and water intake. Consistent with our research, earlier investigations have demonstrated that

alcohol consumption(Kołota *et al*., 2019; Nelson *et al*., 2016) and fructose consumption (Ramos *et al*., 2017) in rats led to decreased food consumption compared to control. Also, rats fed sweetened alcohol (30% alcohol and 30% fructose) in another study had reduced food consumption in comparison to rats fed regular rat chow, fructose only, and ethanol only (Alwahsh *et al.,* 2014). This is an indication that the rats most likely received most of their dietary calories from alcohol and fructose, as it is known that 1g of alcohol (7 kcal) and 1 g of fructose (4 kcal) provide more calories than 1g of the standard rat chow that provides 2.8 kcal (Mamikutty *et al*., 2014). It is believed that appetite regulation in rats is dependent on the number of calories obtained from the food consumed (Menaker & Navia, 1973). This, therefore, probably explains the reduction in food intake seen in rats on sweetened alcohol.

Our study showed that the sweetened alcohol-induced reduction in food intake appeared earlier in male rats compared to female rats. Studies have reported sex differences in alcohol preference in rats. In SD rats, it has been reported that female rats exhibit a greater preference for alcohol and sugar compared to male rats (Nieto & Kosten, 2017). However, alcohol-related consequences such as alcohol use disorder (AUD), which is related to reduced appetite (Kokavec, 2011), are usually reported to affect males more than females (Erol & Karpyak, 2015). This may explain why the sweetened alcohol-induced decrease in food intake was observed earlier in male rats.

Even though it has been shown that alcohol consumption decreases the activity or inhibit the secretion of antidiuretic hormone, making an individual excrete a large amount of urine following alcohol consumption (Harper *et al*., 2018), considering fluid intake, our study reported no sex or group differences in water intake. There was no

difference in water consumption between the sweetened alcohol groups and the control group, showing no evidence of dehydration. This is probably due to the vehicle we used (gelatine), which was made up of greater than 90% water. Therefore, the rats most likely received a significant proportion of their daily water requirements from the gelatine solution used as a vehicle and hence did not need much supplemental drinking water.

4.4.2 Effects of naringin and sweetened alcohol on liver mass

Although liver weight changes are not specifically diagnostic, they can help identify and measure the effects of hepatotoxins (Cattley & Cullen, 2013). Previous studies involving alcohol and fructose reported different results concerning the individual effects of fructose and alcohol on the mass of the liver. Alcohol (Wang *et al.,* 2020), and fructose (Maithilikarpagaselvi *et al*., 2016) administration in Wistar rats resulted in an increased relative liver mass in male Wistar rats, while in another study, female mice administered alcohol had lower liver mass compared to the control (Zhang *et al.,* 2017). However, a different study supported the results from our study, wherein male Wistar rats, after fructose consumption, showed no change in liver mass as compared to the control (Vasiljević *et al*., 2014). Additionally, when male Wistar rats were fed with 66% fructose, there was an increase in the absolute mass of the liver compared to the control (Huang *et al.,* 2016). Thus, demonstrating a possible dose-dependent effect of fructose on the mass of the liver may be possible.

The mass of the liver is strongly linked to nutrition and underlying pathology (Ji *et al*., 2014). The increased mass of the liver can be associated with hepatocyte fat accumulation, hypertrophy, and hyperplasia as adaptive processes in more advanced

liver pathology (Cattley & Cullen, 2013). When the liver was indexed to body weights, naringin in combination with sweetened alcohol led to an increased mass of the liver in male and female rats. This might potentially indicate that supplementation of sweetened alcohol with naringin may pose a risk of increasing liver mass.

However, liver mass alone does not necessarily provide much information about the function and microstructure of the liver. Therefore, histological analysis is necessary, particularly to assess steatosis, which is associated with alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), now referred to as metabolic dysfunction-associated fatty liver disease (MAFLD) (Kulkarni & Sarin, 2023).

4.4.3 Effects of naringin and sweetened alcohol on hepatic steatosis

In agreement with my findings, previous studies have shown that alcohol (Arafa *et al.,* 2019) and fructose (Shawky *et al*., 2019) consumption in male and female rats was associated with hepato-steatosis. In this study, prominent microvesicular steatosis induced by sweetened alcohol consumption in both male and female rats was noted. Microvesicular steatosis affected male and female rats on sweetened alcohol more than other groups. Female rats were more affected in terms of macrovesicular steatosis as shown by the greater macro-steatosis score than the male rats in our study. The female rats had more sweetened alcohol-induced visceral adiposity in our study. Research has indicated that visceral obesity plays a direct role in the development of hepatic steatosis by upregulating the production of adipokines, proinflammatory cytokines, fatty acid formation, increasing triglycerides, and increasing lipogenesis, thereby increasing the inflammatory process leading to hepatic steatosis (Bansal *et al*., 2023; Li *et al*., 2022). Although oestrogen has been shown to confer protection against diet-induced liver pathology (Kamada *et al*., 2024),

vulnerability to liver injury may arise during the developmental and transition stages, like in adolescence, when oestrogen levels fluctuate (Ley *et al*., 1992). In animal studies involving young male and female mice with 'cafeteria diet-induced obesity', it was reported that young female mice developed more severe steatosis compared to their male counterparts (Rodrigues *et al*., 2018), thus further confirming sexual dimorphism in the development of hepatic steatosis.

Naringin improves lipid and ethanol metabolism by reducing the negative consequences of ethanol use on the hepatocytes, demonstrating a hepatoprotective role (Shilpa *et al.,* 2023; Zhou *et al.,* 2019) In a rat model of alcoholic liver disease, naringin at a dosage of 100 mg/day also decreased necrosis, steatosis, and fibrosis as indicated by reduced expression of Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1α / PPARGC1α) (Oliva *et al*., 2008). By reducing hepatic lipogenesis, naringin has been shown to prevent fructose-induced hepatic steatosis in SD rats (Sumarithum *et al*., 2019). In high fructose-fed diabetic rats, naringin prevented hepato-steatosis by significantly reducing the augmentation of oxidative stress, mitochondrial apoptosis, and inflammation caused by the receptor for advanced glycation end-products/ nuclear factor-kB (RAGE/NF-κB) in T2DM-induced steatohepatitis (Ahmed *et al*., 2020). These mechanisms might explain how naringin protected against sweetened alcohol-induced hepatic steatosis in our study. However, future studies should consider exploring the molecular basis of naringin's prevention of sweetened alcohol-induced hepatic steatosis.

4.4.4 Effects of naringin and sweetened alcohol on fasting blood glucose, serum triglyceride, and serum insulin

In this study, naringin supplementation induced increased FBG in female rats, contrary to the findings of most studies, which have reported the hypoglycaemic effects of naringin in both male and female rats (Hartogh & Tsiani, 2019). The study also showed increased serum insulin concentration in male rats on sweetened alcohol supplemented with naringin, while in the female rats, sweetened alcohol and naringin supplementation led to decreased serum insulin, showing a sexually dimorphic response. Despite these changes, the FBG concentration in this study remained within the normal range as reported in rats (3.95 ± 1.3 mmol/L) (Wang *et al.*, 2010). However, the effect of naringin and/or sweetened alcohol might show a potential risk of prediabetes and/or insulin resistance in both male and female rats. Further studies may be required to explore this effect. Sex differences in the effects of naringin on FBG and insulin have not been reported and remain an area for future studies.

Naringin supplementation in combination with sweetened alcohol in females led to a decrease in serum TG concentration. In agreement with this finding, a study involving fructose-fed rats showed that naringin supplementation decreased fructose-induced increase in TG concentration by downregulating the expression of proteins and enzymes involved in TG synthesis (Pengnet *et al.,* 2022), specifically, Glycerol-3-Phosphate Acyltransferase 1 (GPAT-1) and Diacylglycerol/acyl-CoA Acyltransferase-2 (DGAT2). Naringin also downregulated the expression of Carbohydrate Response Element-Binding Protein (ChREBP), Sterol Regulatory Element Binding Protein-1c (SREBP-1c), Nuclear SREBP-1c (nSREBP-1c), Fatty Acid Synthase (FAS), and Acetyl CoA Carboxylase (ACC) (Pengnet *et al.,* 2022). These may likely be similar mechanisms through which naringin, in combination with sweetened alcohol, led to decreased TG concentration in female rats. However, this remains an area to be

explored further since this effect was not seen in male rats. As noted earlier, SOH resulted in an increased macrosteatosis score in female rats, which was prevented by naringin. Thus, the low serum TG concentration in the SOH + NA female rats could be linked to the absence of hepatic macrosteatosis, as the liver is a major source of plasma TGs (Alves-Bezerra & Cohen, 2018).

4.5 Conclusion

To our knowledge, this is the first study that explored the influence of naringin on sweetened alcohol-induced hepatic steatosis in male and female SD rats. We showed that in males and females, sweetened alcohol caused microvesicular hepatic steatosis. In females, sweetened alcohol caused increased visceral fat mass and increased hepatic macrovesicular steatosis. Naringin decreased serum triglycerides and prevented hepatic steatosis in males and females. These results advance our comprehension of the potential metabolic implications of consuming sweetened alcohol, particularly regarding lipid metabolism and liver health. In addition, the findings indicate that female SD rats were more susceptible to developing metabolic derangements associated with sweetened alcohol consumption. Notable sex-related differences in the metabolic responses to the interventions were observed. This finding highlights the need for studies to include both males and females in the study design. Additionally, our findings suggest that naringin may serve as a potential supplement to mitigate the metabolic consequences associated with the consumption of sweetened alcohol. This presents a natural intervention in the fight against MAFLD and consequently reduces the health burden of this condition.

Having explored the effect of the intervention on liver function and other metabolic parameters, the next important organ affected by alcohol and fructose consumption is the kidney. The next chapter focuses on the effects of sweetened alcohol and naringin on kidney function.

CHAPTER FIVE - EFFECTS OF NARINGIN AND SWEETENED ALCOHOL ON EARLY MARKERS OF NEPHROPATHY IN MALE AND FEMALE ADOLESCENT SPRAGUE-DAWLEY RATS

5.1 Introduction

Kidney disease is a major public health concern affecting more than 800,000,000 people globally (Stevens *et al*., 2024), with chronic kidney disease affecting one in ten of the global population (Kovesdy, 2022). By 2040, kidney disease will rank as the sixth most frequent non-communicable disease (NCD) that causes death worldwide due to its persistent growth (Francis *et al*., 2024). Kidney disease lowers quality of life, raises the risk of death, morbidity, and disability, has severe economic repercussions for both the individual and the healthcare system, and has detrimental effects on the environment (Francis *et al*., 2024). The frequency, morbidity, mortality, and costs of chronic kidney disease are all steadily rising, especially in low-income nations (Stevens *et al.,* 2024). Millions suffer from kidney failure and need treatment, such as renal replacement therapy and/or dialysis, but many are unable to get it and die a premature death (Francis *et al*., 2024). Even though studies have reported chronic kidney disease to affect females more than males, end-stage renal disease has been reported to affect males more than females (Garc, 2020.; Harris & Zhang, 2020). Single-sex studies were most frequently used in earlier experimental investigations involving experimental animals to reduce response variability. There is still much to be done to understand the mechanisms related to disparities in gender response to kidney disease (Harris & Zhang, 2020). Excessive alcohol and sugar consumption, among other risk factors, have been linked to an increased risk of developing kidney disease (He *et al*., 2021; Kazancioğlu, 2013).

Alcohol (Latchoumycandane *et al*., 2014) and fructose (Sharma *et al*., 2015) consumption have independently been identified as risk factors for kidney failure. Chronic exposure to alcohol (Latchoumycandane *et al.,* 2014) and excess fructose

(Nakayama *et al*., 2010) can lead to structural impairment and functional changes in the kidneys. An increase in alcohol and fructose consumption is associated with increased formation of inflammatory mediators that may, in turn, lead to kidney injury (Varga *et al*., 2017). The alterations result from both poor microcirculation and the direct harmful effects of excessive concentrations of ethanol and its metabolite, acetaldehyde (Vaidya *et al*., 2010). Additionally, fructose-induced cell proliferation and hyperplasia (Nakayama *et al.,* 2010) in the renal tissue can occur. If not detected early, this can lead to more advanced kidney disease.

The imprecision of currently available indicators (such as urea and creatinine) and their slow response time in the diagnosis of kidney diseases have hindered the ability to detect early renal impairment quickly (Wen & Parikh, 2021). Indicators, such as urea and creatinine, can only be detected when more than half of the renal function is lost (Vaidya *et al.,* 2010), which may delay intervention. There are currently other biomarkers of kidney function that are sensitive and can detect kidney damage before substantial loss of kidney function ((Strauß *et al*., 2024).

Kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are increasingly being used as tubular biomarkers of acute kidney injury, and they can be detected in serum or urine (Pietrukaniec *et al*., 2020). Neutrophil gelatinase-associated lipocalin protein (NGAL), a 25-kDa glycoprotein member of the lipocalin superfamily, was initially found in human-activated neutrophils (Flower, 2000). Under normal circumstances, relatively small amounts of NGAL are expressed and secreted by the kidneys, trachea, lungs, stomach, and colon, and following glomerular filtration, there is almost total reabsorption of NGAL through the mechanism of megalin-mediated endocytosis (Gala-Błądzińska *et al*., 2017). NGAL has also been reported to be elevated in patients with both acute and chronic kidney diseases (Banai

et al., 2021). NGAL increases in both plasma and urine as soon as two hours after the injury and significantly before serum creatinine elevation, which happens 24–48 hours later, making NGAL a very sensitive biomarker of kidney injury (Liang & Shi, 2010).

Kidney injury molecule 1 (KIM-1) is a transmembrane glycoprotein (Liang & Shi, 2010). KIM-1 expression is minimal in healthy kidneys and other organs, but upon injury, it is markedly up-regulated in the proximal tubules of the kidney (Ichimura *et al*., 1998; Liang & Shi, 2010), making it a sensitive and reliable marker for the diagnosis of kidney injury (Castillo-Rodriguez *et al*., 2017).

In some situations, there may be alterations in the markers of renal function; however, important features of renal damage, such as tubular atrophy and interstitial fibrosis, remain hidden. In these types of cases, histological assessment may be of immense benefit to determine the extent of tissue injury and possible effects on renal function (Trevisani *et al*., 2023). Thus, in this study, I assayed for NGAL and KIM-1 and undertook a histological assessment of the kidneys to assess renal health.

There is a shortage of renoprotective medications (Amini *et al*., 2019), such as SGLT2 inhibitors (cenaglitazone, Dapagliflozin), renin-angiotensin system inhibitors (Enalapril, losartan, valsartan) (Ishibashi *et al*., 2023), hence alternative treatment modalities with better outcomes should be explored. Research on the use of naturally occurring plant-derived chemicals as potential therapeutic agents for a variety of disease conditions is a subject of interest. Naringin is a flavonoid in citrus fruits (Bharti *et al.,* 2014). Naringin has a broad spectrum of biological activities, including antioxidant, anti-inflammatory, and renoprotective effects (Amini *et al.,* 2022; Shilpa *et al*., 2023). The potential of naringin in the prevention and treatment of renal diseases has been well-documented (Amini *et al*., 2022; Sehrawat *et al*., 2021). However, its

potential for ameliorating nephropathy and preventing kidney injury induced by the consumption of sweetened alcohol has not been reported. Therefore, this study aimed to explore the potential effects of sweetened alcohol and naringin on the early markers of nephropathy and kidney histopathology in male and female Sprague-Dawley rats.

5.2 Methods

5.2.1 Study settings, study design, animal housing, and ethical approval

As described in chapter three – 3.3.1, 3.3.2 & 3.3.3

5.2.2 Terminal procedures

As described in Chapter Three – 3.3.7

5.2.3 Kidney mass determination

During the terminal procedures, both kidneys were removed and weighed. The right kidney from each rat was preserved in 10% phosphate-buffered formalin and was used for histological examination.

5.2.4 Determination of serum Neutrophil gelatinase-associated lipocalin (NGAL) and Kidney injury molecule-1 (KIM-1)

The stored serum was used to determine the concentrations of NGAL and KIM-1. Serum NGAL concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit [Elabscience ® Rat NGAL (Neutrophil Gelatinase Associated Lipocalin) ELISA kit, Cat. No.: E-EL-R0662. Houston, TX, USA]. For full protocols and procedures for NGAL determination using ELISA see appendix 6A.

Similarly, an ELISA kit [Elabscience ® Rat KIM-1 (kidney injury molecule 1) Cat. No.: E-EL-R3019. Houston, TX, USA] was used to determine the serum KIM-1 concentration. For full protocols and procedures for KIM-1 determination using ELISA see appendix 6B.

5.2.5 Histological assessment of the kidney

An automatic tissue processor (Microm STP 120, Thermo Scientific, MA, USA) was used to process the formalin-fixed kidney samples. Each processed kidney was embedded in paraffin wax, sectioned at 4µm, mounted onto slides, and stained with haematoxylin and eosin (H&E). The slides were viewed under a light microscope [Axioskop 2 microscope; (Leica Biosystems, USA)] with a mounted Axiocam HRc2 camera, which uses ZEISS ZEN microscope software for image capture. The images from the H&E-stained slides were used to assess corpuscular and glomerular tuft areas, and glomerular density under a 40x objective (x100 magnification). The corpuscular area, glomerular tuft area, and glomerular density were assessed using electronic image analysis (ImageJ version 1.54d, Laboratory for Optical and Computational Instrumentation, University of Wisconsin). The assessor was blinded to the rats' identities and treatment groups.

Urinary space area was computed using the formula (Kashif *et al*., 2020):

Urinary space area (μm^2) = Renal corpuscular area (μm^2) – Glomerular tuft area (μm^2) Glomerular density was computed using the following formula (Nigro *et al*., 2018): *Glomerular density ($N/\mu m^2$) =* $\frac{\text{Number of renal corpuscles per section (N)}}{\text{Total area of the section } (\mu m^2).}$

5.2.6 Statistical analysis

Data were analysed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA), and graphs were generated using GraphPad Prism version 9.4 software (GraphPad Software Inc., San Diego, USA). Shapiro-Wilk tests were used for normality tests. All data were normally distributed. Two-way analysis of variance

(ANOVA) followed by Tukey's *post hoc* test was used to analyse kidney masses, kidney morphometry, serum concentrations of NGAL and KIM-1, and sex variations, with group and sex as the main effects. Data were expressed as means ± standard deviation (SD), and $p<0.05$ was considered significant.

5.3 Results

5.3.1 Kidney mass

Table 5.1 shows the effects of sweetened alcohol and naringin on the relative mass of the right and left kidneys in male and female Sprague-Dawley rats. There was no significant difference in the group effect for the relative mass of the right ($F = 1.103$, $p = 0.35$) and left kidney ($F = 2.026$, $p=0.1180$). However, there was a significant difference in the sex effect for the relative mass of the right ($F = 10.59$, $p=0.001$) and left kidney ($F = 34.34$, $p<0.0001$). Compared to male rats in SOH and SOH+NA groups, female rats in SOH ($p=0.0007$) and SOH+NA ($p=0.02$) had higher relative left kidney mass. There was no statistically significant difference in the combined relative mass of the right and left kidneys in both male and female rats ($p>0.05$).

In summary, sweetened alcohol and naringin did not affect the relative mass of the kidneys in male and female rats. Female rats on sweetened alcohol had higher relative kidney masses compared with male rats on sweetened alcohol.

Table 5.1: Effect of sweetened alcohol and Naringin on the relative mass of the kidneys in male and female Sprague Dawley rats.

Parameter	Sex	Group			
		Control	SOH	NA	SOH+NA
Relative right kidney mass (%BM)	Males	0.32 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.30 ± 0.02
	Females	0.32 ± 0.02	0.32 ± 0.02	0.32 ± 0.01	0.32 ± 0.01
Relative Left kidney mass (%BM)	Males	0.32 ± 0.01	0.31 ± 0.01[c]	0.31 ± 0.01	0.30 ± 0.01[d]
	Females	0.33 ± 0.02	0.34 ± 0.02	0.33 ± 0.01	0.32 ± 0.01
Relative mass of the right + left kidneys (%BM)	Males	0.64 ± 0.01	0.60 ± 0.01	0.63 ± 0.01	0.61 ± 0.01
	Females	0.63 ± 0.01	0.65 ± 0.01	0.66 ± 0.01	0.64 ± 0.01

[c]$p<0.05$ compared to female SOH. [d]$p<0.05$ compared to female SOH+NA. Control: 0.5% fructose + 6% gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 19, 9 males, 10 females). BM: Body mass; SOH: sweetened alcohol; NA: naringin; SOH+NA: sweetened alcohol + naringin. Data expressed as means ± SD.

5.3.2 Serum concentration of neutrophil gelatinase-associated lipocalin (NGAL)

Figures 5.1 A and B show the effects of sweetened alcohol and naringin on serum concentration of neutrophil gelatinase-associated lipocalin (NGAL) in male (A) and female (B) Sprague-Dawley rats. There was a significant difference in the group effect ($F = 21.28$, $p<0.0001$), and no difference in the sex effect ($F = 0.3523$, $p=0.5547$) in serum NGAL concentration. Male SOH+NA rats had significantly higher serum NGAL concentrations compared to control and SOH ($p=0.04$, $p=0.001$, respectively). Male SOH rats had significantly lower ($p=0.04$) serum NGAL concentration compared to NA. In female rats, NGAL concentrations were reduced in all groups compared to controls ($p<0.0001$, $p=0.01$, $p=0.001$, respectively). In addition, compared to NA, NGAL concentrations were reduced in SOH rats ($p=0.01$).

In summary, in males, sweetened alcohol consumption resulted in a decreased serum NGAL concentration, while naringin supplementation alone and in combination with sweetened alcohol led to increased serum NGAL concentration. In females, sweetened alcohol, naringin supplementation alone, and in combination with sweetened alcohol caused decreased NGAL concentration.

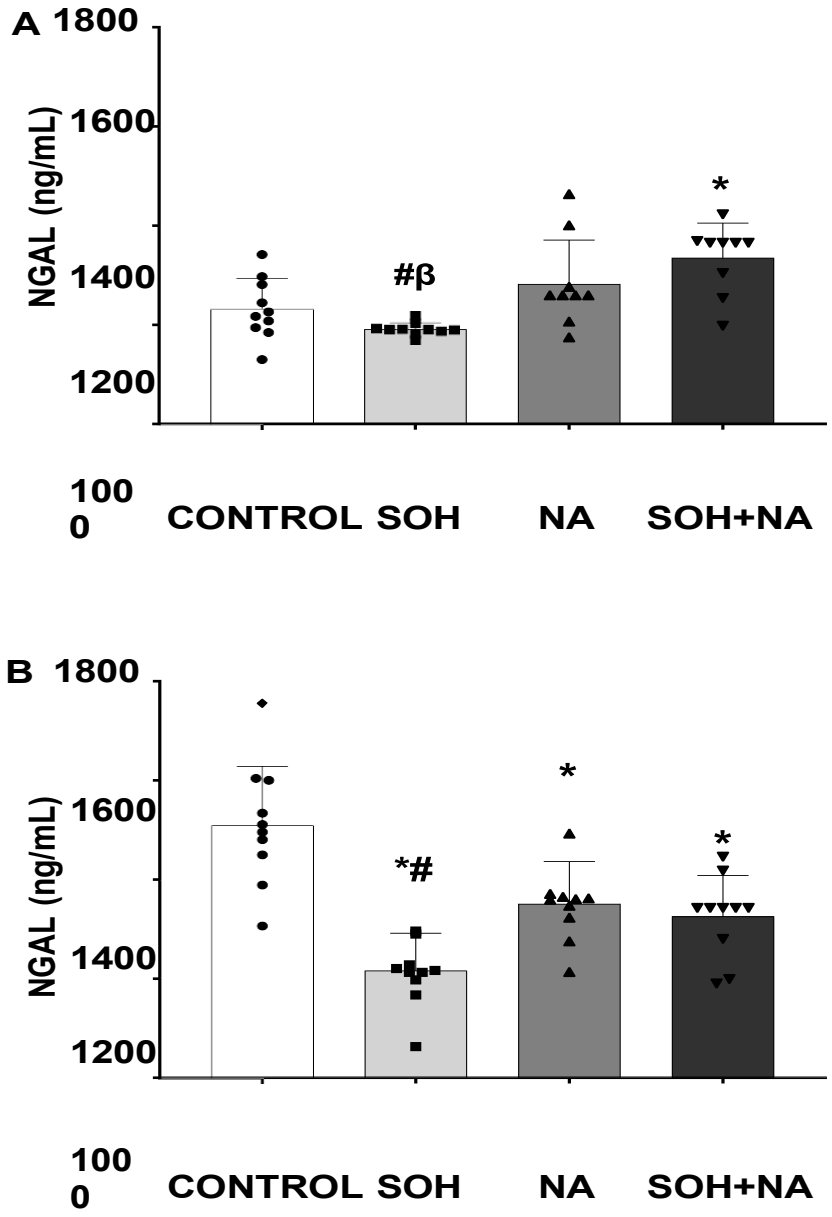

Figure 5.1: Effects of sweetened alcohol and naringin on serum NGAL in male (A) in female (B) Sprague-Dawley rats. Data presented as means ± SD. *p<0.05 compared to control. #p<0.05 compared to NA. βp<0.01 compared to SOH+NA. Control: 0.5% fructose + 6% gelatine at 10ml/100g for 10 weeks (n = 16, 7 males, 9 females). SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 15, 10 males, 5 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 15, 8 males, 7 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in

gelatine at 10ml/100g for 10 weeks (n = 13, 8 males, 5 females). NGAL: Neutrophil gelatinase-associated lipocalin; SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol + Naringin.

5.3.3 Serum concentration of kidney injury molecule 1 (KIM-1)

Figures 5.2 A and B show the effects of sweetened alcohol and naringin on serum concentration of kidney injury molecule 1 (KIM-1) in male (A) and female (B) Sprague-Dawley rats. The group and sex effects were significantly different in serum KIM-1 concentration (group: $F = 26.81$, $p<0.0001$; sex: $F = 20.97$, $p<0.0001$).

In male rats, compared to control and SOH+NA, rats in SOH and NA had a significantly lower serum KIM-1 concentration ($p=0.01$, $p<0.0001$, respectively).

In female rats: compared to control and NA groups, rats in SOH ($p=0.011$; $p=0.002$ respectively) and SOH+NA ($p<0.0001$; $p<0.0001$ respectively) groups had significantly increased serum KIM concentration, and compared to SOH+NA, rats in SOH had significantly decreased serum KIM-1 concentration ($p=0.02$).

Thus, in male rats, consumption of sweetened alcohol led to decreased serum KIM-1, while in female rats, consumption of sweetened alcohol led to increased serum KIM-1 concentrations. Naringin supplementation alone led to decreased serum KIM-1, and in combination with sweetened alcohol, naringin led to increased serum KIM-1.

Compared to female SOH, male rats in SOH had decreased serum KIM-1 concentration ($p<0.0001$), and compared to female SOH+NA, male rats in the SOH+NA group had increased KIM-1 concentration ($p=0.01$).

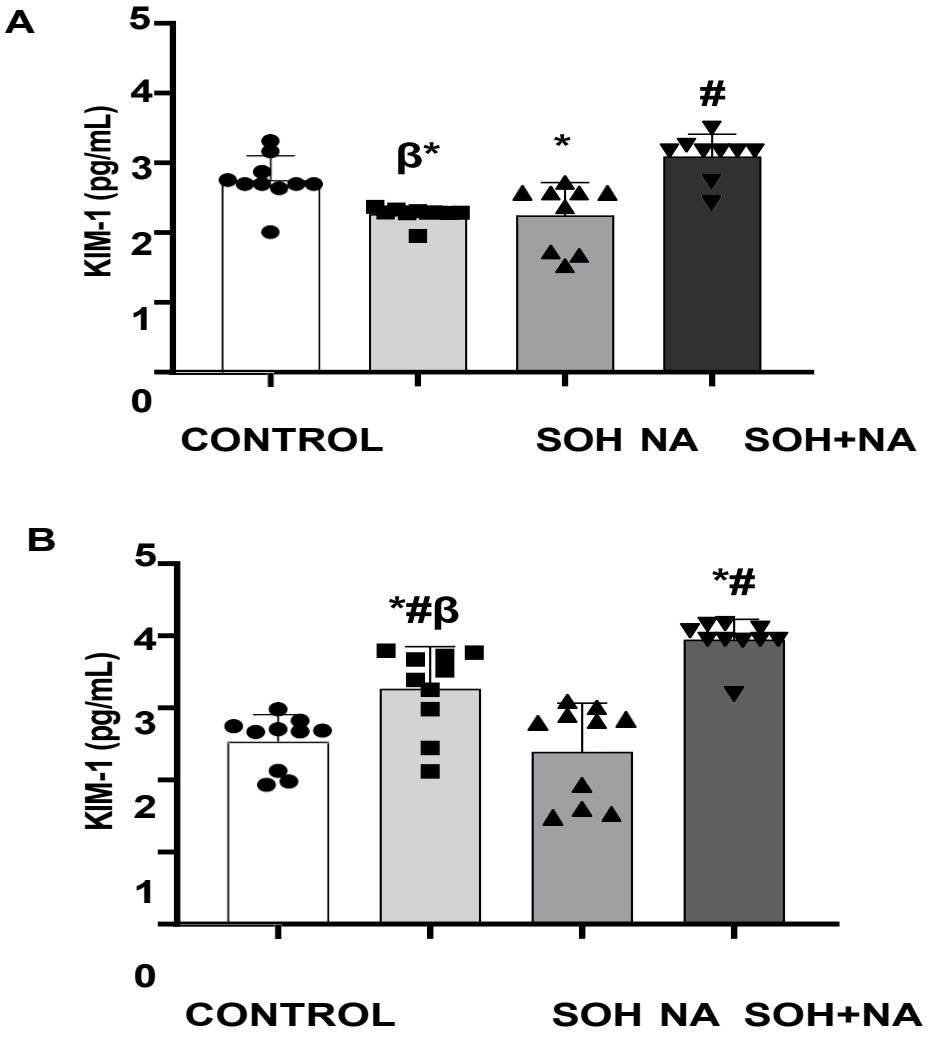

Figure 5.2: Effects of sweetened alcohol and naringin on serum KIM-1 in male (A) in female (B) Sprague-Dawley rats. *p<0.05 compared to control. #p<0.05 compared to NA. βp<0.01 compared to SOH+NA. Control: 0.5% fructose + 6% gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 20, 10 males, 10 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 19, 9 males, 10 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 19, 9 males, 10 females). KIM-1: Kidney injury molecule-1, SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol + Naringin. Data presented as means ± SD, using analysis of variance (ANOVA)

5.3.4 Kidney histomorphometry

Figure 5.3 shows representative photomicrographs of kidney histological sections from male and female Sprague-Dawley rats, respectively. The sections show the corpuscular area (CA), glomerular tuft area (GTA), and urinary space (US) area. Table 5.3 shows the effects of sweetened alcohol and naringin on the kidney corpuscular area, glomerular tuft area, glomerular urinary space, and glomerular density. There were no group and sex effects in the renal corpuscular area in male and female rats (group: $F=3.111.13$, $p=0.034$; sex: $F=10.52$, $p=0.0021$). Compared with female control, male rats in the control group had increased renal corpuscular area ($p=0.03$).

The group and sex effects were significantly different for the glomerular urinary space in male and female rats (group: $F=14.13$, $p<0.0001$; sex: $F=13.47$, $p<0.0001$). In male rats, compared to the control, rats in all the groups had a decreased glomerular urinary space (GUS) ($p<0.0001$), and compared to NA and SOH+NA, rats in SOH had increased GUS ($p=0.0003$; $p=0.0004$, respectively). There was no statistically significant difference in GUS across all the groups in female rats ($p>0.05$). Compared with female control, male rats in the control group had increased GUS ($p=0.02$).

The group and sex effects were also significantly different for the glomerular density in male and female rats (group: $F=4.280$, $p<0.008$; sex: $F=94.16$, $p<0.0001$). In male rats, there was no statistically significant difference in glomerular density across the groups ($p=0.48$). However, in female rats, compared to the NA group, rats in SOH had increased glomerular density ($p=0.005$). Compared to female rats in the control, SOH, and SOH+NA groups, male rats in the control, SOH, and SOH+NA groups had decreased glomerular density ($p<0.0001$; $p<0.0001$; $p=0.009$, respectively).

Thus, in male rats, sweetened alcohol consumption led to decreased glomerular urinary space, and naringin supplementation alone and in combination with sweetened alcohol led to a further decrease in glomerular urinary space. In females, sweetened alcohol and naringin did not affect the glomerular urinary space (see Figure 5.3).

Sweetened alcohol and naringin had no effects on glomerular density in male rats, while in female rats, sweetened alcohol consumption caused increased glomerular density, and naringin did not affect glomerular density.

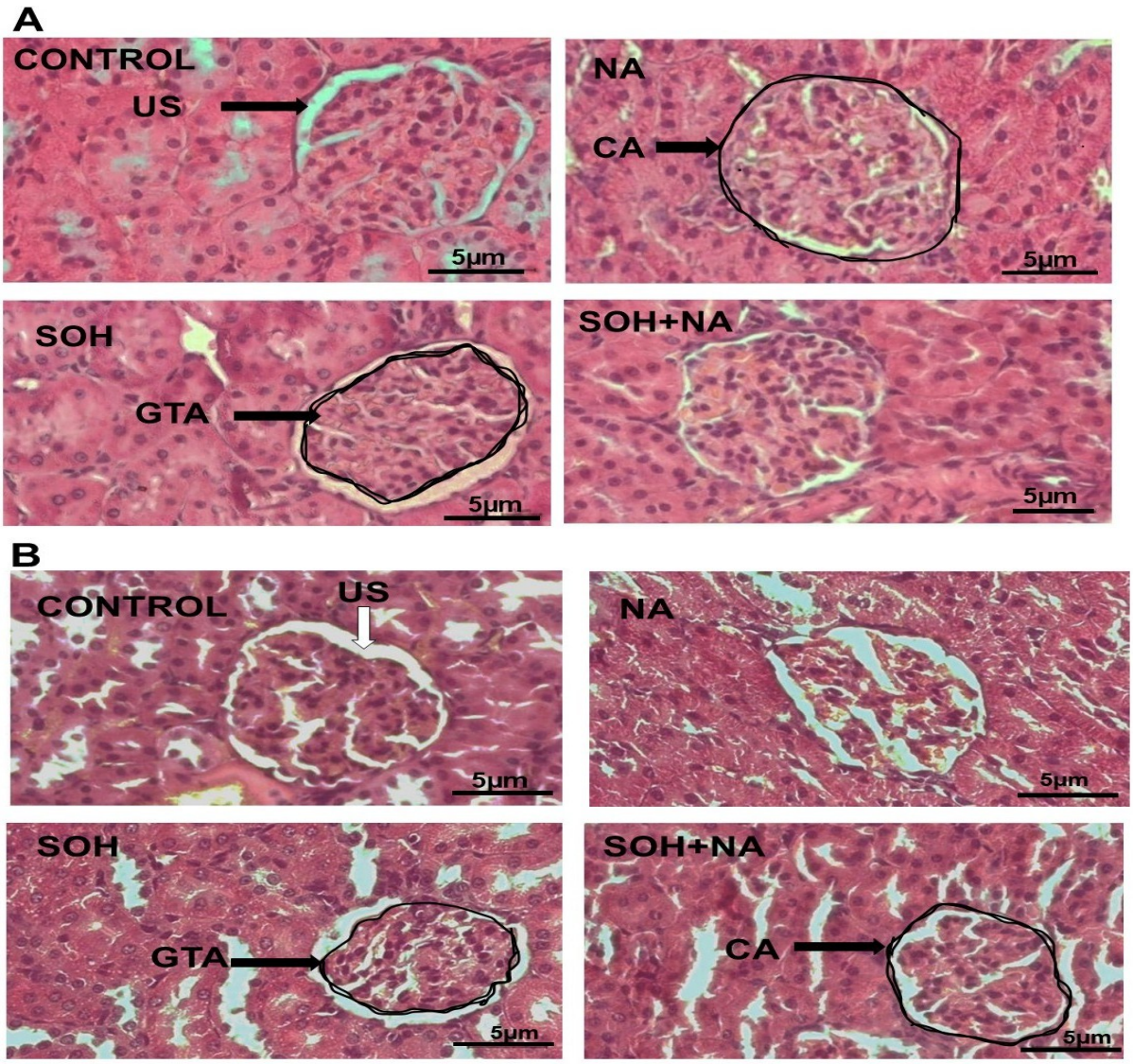

Figure 5.3: Representative photomicrographs of the male (A) and female (B) rat's kidney sections stained with Haematoxylin and Eosin at ×40 objective (x100 magnification). Scale bar = 5µm. Control: 0.5% fructose + 6% gelatine at 10ml/100g for 10 weeks (n = 16, 7 males, 9 females). SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 15, 10 males, 5 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 15, 8 males, 7 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 13, 8 males, 5 females). CA: corpuscular area; GTA: glomerular tuft area; US: urinary space area; NA: Naringin; SOH: Sweetened alcohol; SOH+NA: Sweetened alcohol + naringin.

Table 5.2: Effects of sweetened alcohol and naringin on kidney morphometry in male and female adolescent Sprague-Dawley rats

Parameter	Sex	Group			
		Control	SOH	NA	SOH+NA
Corpuscular area (μm^2)	Males	601.3 ± 57.9***a***	595.8 ± 81.3	582.0 ± 79.9.0	671.6 ± 46.9
	Females	479.0 ± 40.5	564.4 ± 26.3	572.0 ± 129.5	579.6 ± 70.8
Glomerular tuft area (μm^2)	Males	428.2 ± 99.5	470.4 ± 44.8***#a***	560.8 ± 76.9****a***	510.5 ± 54.2***a***
	Females	339.4 ± 40.6	460.3 ± 42.4*	428.5 ± 86.7	412.0 ± 92.0*
Glomerular urinary space area (μm^2)	Males	157.0 ± 8.4***ad***	148.9 ± 21.0****#βcd***	120.8 ± 1.7*	121.5 ± 3.6*
	Females	132.0 ± 28.1	131.4 ± 5.6	122.9 ± 10.6	105.5 ± 0.7
Glomerular density (10^{-4} N/μm^2)	Males	4.1 ± 0.5***abd***	4.4 ± 0.6***abd***	4.5 ± 0.8***abd***	4.5 ± 0.7***abd***
	Females	6.2 ± 1.1	7.5 ± 0.7#	5.5 ± 0.7	6.0 ± 0.1

*p<0.05 compared to control. #p<0.05 compared to NA. ***β***p<0.01 compared to SOH+NA. ***a***p<0.05 compared to the female control.p<0.05 compared to female SOH. ***c***p<0.05 compared to female NA. dp<0.05 compared to female SOH+NA. Control: 0.5% fructose + 6% gelatine at 10ml/100g for 10 weeks

(n = 16, 7 males, 9 females). SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 15, 10 males, 5 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 15, 8 males, 7 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 13, 8 males, 5 females). N: Number, SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol + Naringin. Data expressed as means ± SD.

5.4 Discussion

This study investigated the effects of voluntarily consumed sweetened alcohol and naringin on renal morphology and markers of nephropathy in male and female Sprague-Dawley rats.

Sweetened alcohol and naringin had no effects on the relative mass of the kidneys in male and female rats. In male rats, sweetened alcohol consumption led to decreased glomerular urinary space, and naringin alone and, in combination with sweetened alcohol, induced a greater decrease in glomerular urinary space, while in females, sweetened alcohol and naringin had no effect on glomerular urinary space. Sweetened alcohol did not affect glomerular density in male rats, while in female rats, sweetened alcohol consumption led to increased glomerular density, and naringin had no effects on glomerular density in both male and female rats.

Male rats on sweetened alcohol alone showed decreased serum NGAL, while naringin supplementation alone, and in combination with sweetened alcohol, led to increased serum NGAL. In females, sweetened alcohol consumption, naringin supplementation alone, and in combination with sweetened alcohol caused decreased serum NGAL, with the decrease seen more with rats on sweetened alcohol alone. Likewise, in male rats, sweetened alcohol consumption alone and naringin supplementation alone led to a decrease in serum KIM-1, while naringin supplementation in combination with sweetened alcohol led to increased serum KIM-1 concentration. In females, sweetened alcohol alone and in combination with naringin led to increased serum KIM-1 concentration, while naringin supplementation alone led to decreased serum KIM-1. Each of these findings is analysed and discussed below.

5.4.1 Kidney mass

Assessment of the mass of an organ is important for toxicological assessment of interventions and can indirectly be used to determine organ atrophy or hypertrophy/hyperplasia (Lazic, Semenova, and Williams, 2020). Variations in kidney weight could be a sign of chronic progressive nephropathy, renal toxicity, or tubular hypertrophy (Sellers *et al*., 2007). Diet, and specifically fructose, can have an impact on kidney mass. However, this seems to be dose-dependent, such that high fructose (60% fructose or more) consumption was reportedly associated with increased weight of the kidneys in rats (De Castro *et al*., 2013). However, in line with our study findings, rats fed with 20% fructose showed no difference in the mass of the kidney relative to body mass compared to the control (Fakhoury-Sayegh *et al*., 2019).

Studies investigating the impact of alcohol consumption on kidney mass have shown different results. For example, a study reported an increase in kidney mass in a dose-dependent manner compared with control in rats fed with 5%, 10%, and 12% ethanol (Tehoua *et al*., 2013). A different study reported that rats administered 10% ethanol showed no kidney mass difference compared to the controls (Brzóska *et al*., 2003).

These studies supported our findings that 20% fructose and 10% alcohol consumption did not induce increased relative mass of the kidneys. Kidney mass alone does not provide adequate information on the toxicological assessment of the kidney. Histological assessment can provide a detailed microstructure of the kidney and any possibility of kidney injury, and therefore, we performed a histological examination of the study rats' kidneys.

5.4.2 Kidney Histophotometry

Indeed, histology of the renal tissue has been validated as a useful and reliable tool for the diagnosis of different renal diseases and can detect clinically non-detected renal diseases (Comai *et al*., 2019; Jin & Xie, 2020). Assessment of glomerular morphometry is considered an important means for the diagnosis of renal pathologies (Kashif *et al.,* 2020). Our study showed a decreased glomerular urinary space with consumption of sweetened alcohol, and supplementing with naringin, both alone and in combination with sweetened alcohol, further reduced the glomerular urinary space in male rats. Decreased glomerular urinary space has been identified as an early histopathological change of nephropathy, and it is related to decreased glomerular filtration (Sasaki *et al*., 2018), which may eventually lead to renal failure (Cui *et al*., 2018).

Sweetened alcohol consumption, therefore, induced an early histological sign of nephropathy presenting as decreased glomerular urinary space. However, naringin did not avert the effect, but rather it further decreased the glomerular urinary space. Naringin might have induced a decreased glomerular urinary space as a compensatory mechanism to a decreasing glomerular filtration, as a reduction in glomerular urinary space has been associated with glomerular hypertrophy (Haruhara *et al*., 2019), and glomerular hypertrophy is believed to be a compensatory mechanism for a decrease in glomerular filtration (Kanzaki *et al*., 2017; Tan *et al*., 2009). A metabolite of naringin (naringenin) was reported to improve histological changes in high-cholesterol diet-fed rats by decreasing the increased glomerular urinary space induced by a high-cholesterol diet (Chtourou *et al*., 2016). In the current study, glomerular density was also assessed because of its relationship with glomerular space in kidney function.

This study has revealed that sweetened alcohol consumption alone was associated with increased glomerular density and increased glomerular tuft area in female rats, while, on the contrary, naringin supplementation alone or in combination with sweetened alcohol decreased glomerular density. Reduced glomerular density may be a precursor to an early stage of elevated risk of parenchymal injury and is linked to a variety of renal function and metabolic risk factors (Rule *et al*., 2011). In this regard, sweetened alcohol consumption in females might have beneficial effects against decreased glomerular density, while naringin did not have an effect. It has been reported that low to moderate alcohol consumption can reduce the risk of kidney injury (Li *et al.,* 2022).

The histomorphometry features seen in this study require further evaluation. Thus, biological markers of kidney function and /or injury were also assessed to determine kidney function.

Serum creatinine levels are commonly used to diagnose acute kidney disease in modern clinical practice. However, creatinine is an unreliable marker for the early detection of renal diseases (Devarajan, 2014), because it lacks the necessary sensitivity and specificity to identify renal toxicity before a substantial decline in renal function (Oh, 2020). NGAL and KIM-1 are proposed as more reliable biomarkers for acute kidney damage that is associated with tubular injury (Castillo-Rodriguez *et al.,* 2017).

It has also been reported that measuring the two biomarkers (NGAL and KIM-1) gives better diagnostic accuracy than measuring one of them (Pietrukaniec *et al.,* 2020).

5.4.3 Serum NGAL and KIM-1

5.4.3.1 Serum NGAL

The current study discovered that sweetened alcohol led to decreased serum NGAL concentrations in male and female rats, but sweetened alcohol in combination with naringin led to increased serum NGAL concentrations in males. In females, naringin supplementation alone and in combination with sweetened alcohol showed a decrease in serum NGAL concentrations. Therefore, consumption of sweetened alcohol may offer protection against early kidney injury as shown by the reduction in the concentration of serum NGAL. Even though the literature has not specifically reported findings regarding low to moderate alcohol consumption and serum NGAL concentrations, it has been reported that moderate alcohol consumption protects against kidney injury (Li *et al.,* 2022).

Notably, naringin in combination with sweetened alcohol may be nephrotoxic in male rats, and nephroprotective in female rats. This sexually dimorphic effect could be due to the impact of oestrogen in females. By facilitating the synthesis and release of antioxidants, increasing the activity of nitric oxide synthase enzyme, and enhancing the synthesis, secretion, and regulation of growth factors, cytokines, and vasoactive compounds, oestrogen confers some protection against kidney injury (Güzel *et al*., 2019; Harris & Zhang, 2020).

However, NGAL is not specific to renal tissue as it is also produced by the lungs, the colon, and the stomach (Gala-Błądzińska *et al.,* 2017). Therefore, serum KIM-1 was also measured.

5.4.3.2 Serum KIM-1

From this study, sweetened alcohol consumption alone and naringin supplementation alone led to decreased serum KIM-1 in males, while in females, sweetened alcohol

alone led to increased serum KIM-1 concentration. However, sweetened alcohol in combination with naringin led to an increase in serum KIM-1 in both males and females. Thus, consumption of sweetened alcohol at concentrations used in this study may also protect against increased serum KIM-1 and therefore, protect against kidney injury in males. Also, naringin supplementation alone may protect against kidney injury. However, naringin supplementation in combination with sweetened alcohol may be nephrotoxic in both male and female rats.

A transmembrane protein, KIM-1 is elevated in response to proximal tubular injury (Claudel *et al*., 2024)(. It is hardly detectable in healthy individuals, but patients suffering from acute tubular necrosis have significantly elevated levels of KIM-1 (Pietrukaniec *et al*., 2020). Also, in a mouse experimental model of kidney disease, it has been reported that KIM-1 concentrations were high in both acute and chronic renal disease (Sabbisetti *et al*., 2013). This study reported a sexually dimorphic effect for KIM-1 in that female rats had an increased level, and males had a decreased level of KIM-1 in response to sweetened alcohol consumption. This finding can be supported by another study on sex differences in kidney KIM-1 gene expression, which reported that female rats had increased gene expression for KIM-1 compared to males (Kwekel *et al*., 2013).

Overall, consumption of low to moderate sweetened alcohol may be nephroprotective in both males and females, as it has led to decreased concentration of NGAL in male and female rats and decreased KIM-1 in male rats. Even though there is a paucity of information about the relationship between low to moderate alcohol consumption with NGAL and KIM-1, other traditional biomarkers of kidney function such as serum creatinine and eGFR, have shown a renoprotective effect and a decreased risk of renal dysfunction with moderate alcohol consumption (Schaeffner *et al*., 2005).

The effects of low concentrations of sweetened alcohol seen from this study agree with other studies (Chen *et al*., 2021; Kaartinen *et al*., 2009; Lee *et al*., 2021) that reported that low to moderate alcohol consumption reduces the risk of kidney injury and that alcoholic nephropathy was associated with chronic long-term alcohol consumption (Elnagar *et al*., 2021). Those studies (Nakayama *et al.,* 2010; Sharma *et al.,* 2015), which reported and associated fructose consumption with an increased risk of kidney injury, used high doses (≥60%) of fructose as against the 20% used in our study, which may explain the findings in those studies. Naringin supplementation alone may be nephroprotective. Naringin has been reported to be a nephroprotective agent through its anti-inflammatory and possible scavenging actions of free radicals (Amini *et al.,* 2022). Naringin also protects the kidneys and ameliorates kidney injury by activating the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway and decreasing pro-inflammatory factors like *Cyclooxygenase-2 (*COX-2), tumour necrosis factor-alpha (TNF-α), *Inducible nitric oxide synthase* (iNOS), and apoptotic agents (Amini *et al.,* 2022). However, naringin supplementation with sweetened alcohol showed a nephrotoxic potential, possibly related to the sweetened alcohol model used in this study. The mechanism through which sweetened alcohol supplemented with naringin causes increased expression of early markers of nephropathy remains an area to be investigated in future studies.

In male rats, the histological findings associated with sweetened alcohol supplemented with naringin showed a decreased glomerular urinary space correlated with increased serum concentration of NGAL and KIM-1. This may confirm the Possible nephrotoxic effects associated with sweetened alcohol supplemented with naringin in male rats.

Histological changes of early signs of nephropathy seen with consumption of sweetened alcohol, as against the sweetened alcohol nephroprotection seen from

serum markers of kidney injury, may not be surprising because it has been reported that early histological signs of nephropathy are present in a considerable percentage of people without impacting the tubules or the interstitium around them (Comai *et al.,* 2019).

This study highlighted the possible sexually dimorphic effects of sweetened alcohol consumption and naringin supplementation on acute kidney injury and markers of early kidney injury. Sweetened alcohol led to a decreased terminal body mass and glomerular urinary space in males with no effects in females. It also led to decreased serum KIM-1 in males and an increase in females. Sweetened alcohol led to increased glomerular density in females with no effect in male rats. Naringin supplementation alone and in combination with sweetened alcohol led to increased serum NGAL in males and decreased serum NGAL in female rats. This study demonstrated the potential renoprotective effects of sweetened alcohol consumption at the concentration used in this study, and that naringin supplementation alone may also be a potential supplement to protect the kidneys in both males and females. However, naringin supplementation in combination with sweetened alcohol may be nephrotoxic depending on sex and the serum marker studied.

The next chapter looked at the supplementary data not included in the previous chapters.

CHAPTER SIX – SUPPLEMENTARY FINDINGS: EFFECTS OF NARINGIN AND SWEETENED ALCOHOL ON ERYTHROCYTE INDICES, GIT MORPHOMETRY AND TROPONIN-I IN SPRAGUE-DAWLEY RATS

6.1 Introduction

Measurement of organ mass and morphometry is an important indicator of the possible toxicity of test substances (Sellers *et al.,* 2007). Organ weight variations are frequently measured for novel compounds or other chemical entities and are recognised as a sensitive indicator of chemically induced effects on organs (Lazic *et al*., 2020). Alteration in the weight of the GIT organs can serve as a sensitive indicator to determine an ongoing pathology induced by an exogenous substance (Girgiri *et al*., 2015). Reduction in the weight of the pancreas or pancreatic atrophy can affect its endocrine and exocrine functions (Caglar *et al*., 2014). Weight increases in the testes could be a sign of interstitial oedema or alterations in the seminiferous tubules. Variations in epididymal weight could be a sign of inflammation or oedema, or they could be a sensitive predictor of decreased sperm production (Sellers *et al.,* 2007).

Alcohol and fructose metabolism primarily occur in the liver (Helsley *et al*., 2020). However, when consumed orally, the first point of contact with dietary constituents is the GIT. When consumed in excess, alcohol and fructose can alter the structure and functions of the GIT (Asuzu Onyeka *et al*., 2015; Helsley *et al*., 2020), which can affect the general health status of an individual.

The erythrocyte and its different measurements and indices, particularly the blood's total haemoglobin concentration and haematocrit, are one component of body physiology that is considered a sign of good health, and determines health status (Johnstone *et al*., 2017). When evaluating haematotoxicity, a rise or fall in blood variables suggests either an excess or a suppression of the production of blood cells or an imbalance between the destruction and synthesis of blood cells (Saka 2011). Analysing haematological indices is essential for determining how harmful exogenous substances, such as medicinal herbs, are to the body. After being exposed

to a plant extract, an alteration in red cell indices indicates the extract's antihaematinic potential, or ability to cause anaemia (Yakubu *et al.,* 2007). Independently, fructose and alcohol consumption have been reported to cause alteration of some haematological indices such as the packed cell volume (PCV), haemoglobin (Hb), and mean corpuscular haemoglobin concentration (MCHC), which results in an altered physiological state (Altuğ *et al*., 2024; Eyong *et al*., 2009; Lugos *et al*., 2018).

Circulating cardiac biomarkers are quantifiable indicators that provide insight into cardiac pathophysiology. Troponin I is a contractile protein released by the cardiac muscles in direct proportion to the extent of tissue damage and myocyte membrane rupture (O'Brien *et al*., 2006). Concentrations of circulating cardiac troponin are becoming a more significant biomarker for the non-invasive identification of myocardial damage in various cardiovascular diseases (Ni & Wehren, 2018). Alcohol (Csengeri *et al*., 2021), and fructose (Adoga *et al*., 2021) consumption has been linked to raised cardiac troponin I in rats, which may affect the general function of the heart. Therefore, as an additional measurement to assess the impact of the interventions on the heart, serum troponin-I concentrations were assayed; the data were not included in chapter 3 as the assays were done after the publication of the findings in chapter 3. Hence, the inclusion of troponin-I in this chapter.

Previous ethnopharmacological studies have associated naringin with improved cardiometabolic health (Haș *et al*., 2023). Naringin, through its anti-inflammatory and free radical scavenging potentials, has been shown to protect the small and large intestines and the pancreas against various insults and disease conditions, thereby promoting their physiological role (Ahangarpour *et al*., 2018; Amaro *et al*., 2009; Cerkezkayabekir *et al*., 2017). Naringin increased testicular function by reversing the Bisphenol A-induced decrease in the weight of the testes and improved the

reproductive function of the testes (Alboghobeish *et al*., 2019). Naringin has also been reported to improve the diabetic-induced decrease in haematological parameters such as PV, Hb, and MCHC concentrations in experimental rats (Mahmoud, 2013) Naringin has also been reported to cause a decrease in the concentration of cardiac troponin in isoproterenol-induced myocardial infarction (Rajadurai & Stanely Mainzen Prince, 2007). However, the role of naringin and sweetened alcohol on these organs, serum, and haematological parameters has not been explored.

6.2 Methods

6.2.1 Study settings, study design, animal housing, and ethical approval

As described in chapter three – 3.3.1, 3.3.2 & 3.3.3

6.2.2 Terminal procedures and measurements

As described in chapter three – 3.3.7

6.2.2.1 Erythrocyte indices

Using a drop of blood collected following the cardiac puncture, haemoglobin (Hb) and packed cell volume (PCV) were determined using a veterinary haematocrit-haemoglobin meter (Insight HCT meter, Woodley Equipment Company Ltd, UK) based on the manufacturer's instructions.

The values for Hb and PCV were then used to compute the mean corpuscular haemoglobin concentration (MCHC) as follows (Rahman *et al*., 2020):

MCHC (g/dL) = [Hb (g/dL) ÷ PCV (%)] × 100

Where **MCHC** = Mean corpuscular haemoglobin concentration, **Hb** = haemoglobin, **PCV** = packed cell volume.

6.2.2.2 Cardiac troponin I measurement

The stored serum from five rats, randomly selected per group, was used to determine the concentration of cardiac troponin I. Serum troponin was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Rat TNN13/CTn-1, Troponin1 type 3, cardiac, Cat number: E-EL-R 1253), according to the manufacturer's instructions. According to the manufacturer's specifications, the kit had a sensitivity of 18.75 pg/mL and a detection range of 31.25-2000pg/mL.

6.2.2.3 GIT morphometry and tissue masses

The pancreas, stomach, small intestine, large intestine, caecum, testes, and epididymal fat pads (for males) were dissected out as part of the terminal procedures. The testes were separated from the surrounding epididymal fat, and epididymis. The contents of the stomach and the small and large intestines were emptied. The masses of all dissected tissues were determined using a Presica 310M balance (Precision instrument, Johannesburg, South Africa). The lengths of the small and large intestines were measured using a ruler after gently stretching them on a dissection board.

6.2.3 Statistical analysis

Data were analysed using a Two-way analysis of variance (ANOVA) using GraphPad Prism version 9.4.9 (GraphPad Software Inc., San Diego, USA). Data were expressed as mean ± standard deviation. A multiple comparisons Tukey *post hoc* test was used to compare the means. A two-way ANOVA was also used to compare the effect of sex interactions and treatment variables. A p-value <0.05 was considered significant.

6.3 Results

6.3.1 Erythrocyte indices

Table 6.1 shows the effects of sweetened alcohol and naringin on packed cell volume, haemoglobin, and the mean corpuscular haemoglobin concentration in male and

female Sprague-Dawley rats. There are no group and sex differences in all the parameters in both male and female rats (All $p>0.05$).

Table 6.1: Effects of sweetened alcohol and naringin on haematological parameter in male and female Sprague-Dawley rats

		CONTROL	SOH	NA	SOH+NA
PCV (%)	Males	59.00 ± 6.1	62.0 ± 4.0	63.0 ± 5.0	60.1 ± 3.0
	Females	61.0 ± 4.0	58.0 ± 5.0	58.0 ± 5.0	61.0 ± 4.4
Hb (g/dL)	Males	20.0 ± 2.1	21.0 ± 1.3	20.7 ± 1.6	20.1 ± 0.8
	Females	20.3 ± 1.3	19.2 ± 1.7	19.2 ± 1.6	20.7 ± 0.6
MCHC (g/dL)	Males	33.3 ± 0.1	33.3 ± 0.2	32.9 ± 1.1	33.4 ± 0.1
	Females	33.4 ± 0.1	33.2 ± 0.5	33.3 ± 0.2	34.0 ± 1.8

Data expressed as mean ± standard deviation. PCV: Packed cell volume; Hb: Haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; SOH: sweetened alcohol, NA: Naringin; SOH+NA: Sweetened alcohol and naringin

6.3.2 Cardiac troponin I

Figure 6.1 shows the effects of sweetened alcohol and naringin on the serum concentration of cardiac troponin I in male and female Sprague-Dawley rats. There were no group or sex differences in the serum concentration of cardiac troponin I in male and female Sprague-Dawley rats (all $p>0.05$).

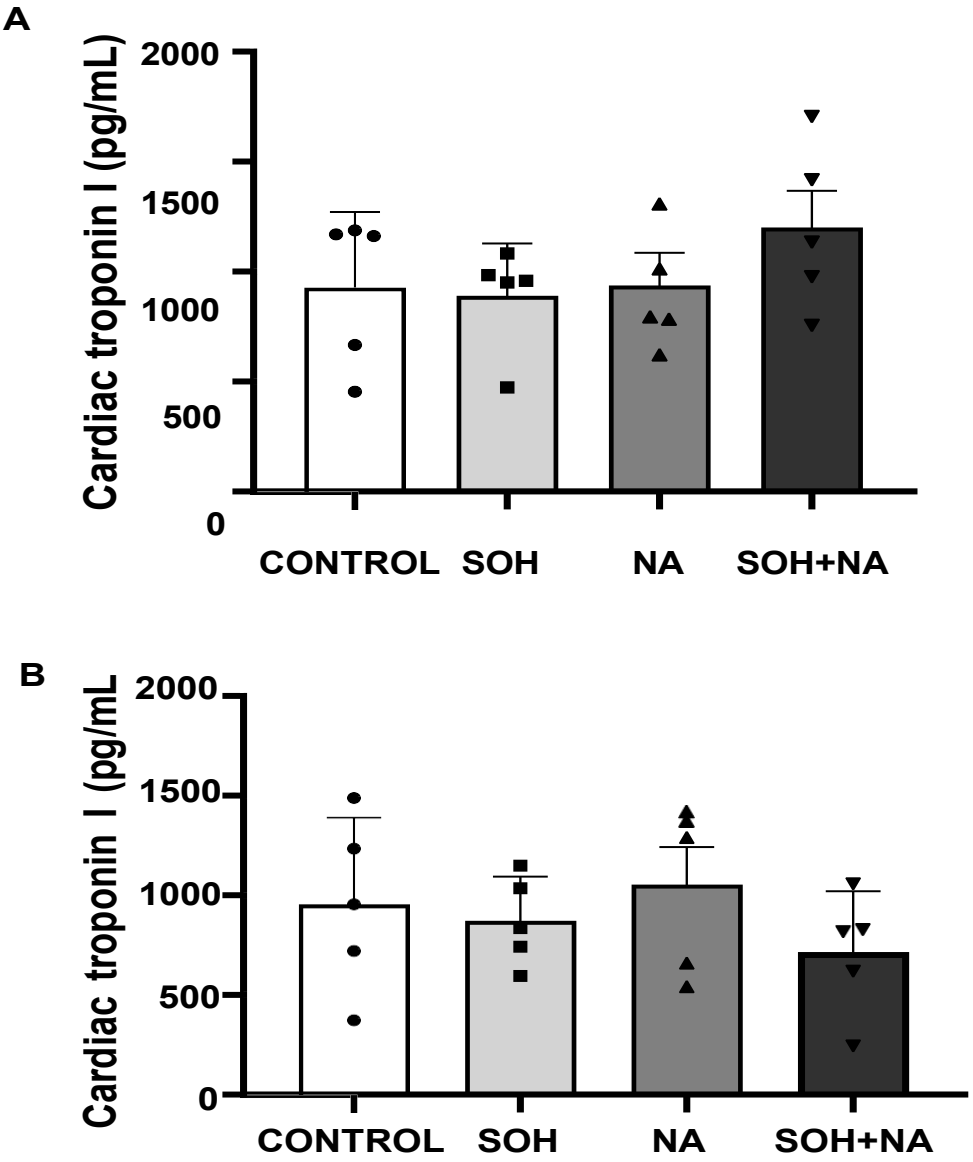

Figure 6.1: Effects of sweetened alcohol and naringin on serum cardiac troponin I in male (A) and female (B) Sprague-Dawley rats. Control: 0.5% fructose in 6% gelatine at 10ml/100g for 10 weeks (n = 10, 5 males, 5 females); SOH: 20% fructose + 10% ethanol in gelatine at 10ml/100g for 10 weeks (n = 10, 5 males, 5 females). NA: 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 10, 5 males, 5 females). SOH+NA: 20% fructose + 10% ethanol + 50mg/kg naringin in gelatine at 10ml/100g for 10 weeks (n = 10, 5 males, 5 females). SOH: Sweetened alcohol; NA: Naringin; SOH+NA: Sweetened alcohol and naringin. Data presented as means ± SD.

6.3.3 Visceral Organs Gross Morphometry

Table 6.2 shows the effects of sweetened alcohol and naringin on visceral organ morphometry in male and female Sprague-Dawley rats. There were no group differences in all the parameters in both male and female rats (all $p>0.05$). However, compared with male control and SOH+NA, female rats in the corresponding groups had decreased absolute pancreatic mass ($p<0.0001$, $p=0.01$ respectively), while when indexed to body mass, male rats in SOH and NA had decreased pancreatic mass compared to female rats from the corresponding groups ($p=0.009$, $p<0.0001$ respectively). Male rats in the control, NA, and SOH+NA groups had significantly increased stomach absolute mass compared to female rats from the corresponding groups ($p<0.0001$), while male rats in the control, SOH, NA, and SOH+NA groups had decreased stomach mass compared to female rats from the corresponding groups ($p<0.0001$). When indexed to body mass, compared to female control, NA, SOH, and SOH+NA, male rats from the corresponding groups had significantly increased absolute mass of the caecum ($p<0.0001$, $p=0.0003$, $p<0.0001$, $p<0.0001$, respectively). Compared to female rats in the control, NA, SOH, and SOH+NA, male rats in the corresponding groups had significantly increased absolute mass of the small intestine ($p<0.0001$). When indexed to body mass, female rats in the control, NA, SOH, and SOH+NA groups had increased mass of the small intestine compared to male rats in the corresponding groups ($p<0.0001$). Female rats in the control, NA, SOH, and SOH+NA groups had decreased length of the small intestine compared with male rats in the corresponding groups ($p<0.0001$, $p=0.005$, $p<0.0001$, $p<0.0001$, respectively). For the mass and length of the large intestine, compared to female control, SOH, and SOH+NA, male rats in the corresponding groups had significantly increased absolute mass of the large intestine ($p<0.0001$, $p=0.005$, $p<0.0001$, $p<0.0001$, respectively).

When indexed to body mass, compared to male rats in SOH, NA, and SOH+NA, female rats from the corresponding groups had a significant increase in mass of the large intestine ($p=0.03$, $p=0.006$, $p=0.02$, respectively). Compared to female NA, male rats in the NA group had significantly increased length of the large intestine ($p=0.03$).

Table 6.2: Effects of sweetened alcohol and naringin on visceral organs gross morphometry in male and female SD rats

Parameter	Sex	CONTROL	SOH	NA	SOH+NA
Pancreas (g)	**Males**	1.56 ± 0.18[a]	1.41 ± 0.41	1.33 ± 0.18	1.47 ± 0.28[d]
	Females	1.04 ± 0.19	1.17 ± 0.18	1.11 ± 0.20	1.10 ± 0.11
Pancreas (%BM)	**Males**	0.33 ± 0.05	0.32 ± 0.08[b]	0.29 ± 0.04[c]	0.34 ± 0.06
	Females	0.40 ± 0.06	0.42 ± 0.06	0.43 ± 0.07	0.41 ± 0.05
Stomach (g)	**Males**	1.82 ± 0.16[a]	1.71 ± 0.25	1.87 ± 0.09[c]	1.89 ± 0.19[d]
	Females	1.44 ± 1.42	1.54 ± 1.16	1.38 ± 1.23	1.41 ± 1.16
Stomach (%BM)	**Males**	0.39 ± 0.02[a]	0.39 ± 0.06[b]	0.41 ± 0.05[c]	0.44 ± 0.03[d]
	Females	0.56 ± 0.07	0.56 ± 0.04	0.53 ± 0.03	0.52 ± 0.05
Caecum	**Males**	1.59 ± 0.24[a]	1.38 ± 0.17[b]	1.60 ± 0.21[c]	1.46 ± 0.14[d]
	Females	1.05 ± 0.16	1.07 ± 0.15	1.09 ± 0.18	1.12 ± 0.17
Caecum (%BM)	**Males**	0.34 ± 0.05	0.32 ± 0.04	0.35 ± 0.05	0.34 ± 0.05
	Females	0.40 ± 0.05	0.39 ± 0.04	0.42 ± 0.06	0.42 ± 0.08
Small Intestine (g)	**Males**	9.09 ± 0.82[a]	8.47 ± 0.79[b]	9.16 ± 0.66[c]	8.72 ± 0.96[d]

	Females	6.71 ± 0.89	6.97 ± 0.89	6.65 ± 0.46	6.90 ± 0.36
Small Intestine (%BM)	**Males**	1.95 ± 0.12	1.93 ± 0.16	1.99 ± 0.18	2.04 ± 0.14
	Females	2.57 ± 0.28	2.53 ± 0.13	2.56 ± 0.07	2.55 ± 021
Length of Small Intestine (mm)	**Males**	1374.00 ± 59.90**a**	1319.00 ± 62.36[b]	1394.00 ± 39.17[c]	1363.00 ± 54.84**d**
	Females	1203.00 ± 78.89	1220.00 ± 29.86	1213.00 ± 64.73	1228.00 ± 47.57
Large Intestine (g)	**Males**	1.86 ± 0.23[a]	1.80 ± 0.44**b**	1.88 ± 0.21**c**	1.74 ± 1.45
	Females	1.32 ± 0.24	1.37 ± 0.13	1.35 ± 0.12	1.47 ± 0.20
Large Intestine (%BM)	**Males**	0.40 ± 0.03	0.41 ± 0.08**b**	0.41 ± 0.05**c**	0.41 ± 0.04d
	Females	0.51 ± 0.09	0.50 ± 0.05	0.52 ± 0.03	0.54 ± 0.07
Length of Large Intestine (mm)	**Males**	220.00 ± 18.56	209.50 ± 12.79	221.10 ± 17.64**c**	219.40 ± 12.61
	Females	198.50 ± 9.14	194.40 ± 13.33	197.50 ± 11.37	199.50 ± 23.97
Testes	**Males**	5.89 ± 0.44	5.71 ± 0.50	5.84 ± 0.53	5.76 ± 0.47
Testes (%BM)	1.26 ± 0.06	1.30 ± 0.08	1.14 ± 0.41	1.35 ± 0.13	1.26 ± 0.06
Epididymal fat	**Males**	3.01 ± 0.49	2.74 ± 0.40	2.94 ± 0.55	2.57 ± 0.73
Epididymal fat (%BM)	0.64 ± 0.09	0.62 ± 0.09	0.57 ± 0.23	0.59 ± 0.14	0.64 ± 0.09

Data expressed as mean ± standard deviation. [a]p<0.05 compared to female control. [b]p<0.05 compared to female SOH. [C]p<0.05 compared to female NA. [d]p<0.05 compared to female SOH+NA. BM: Body mass, g: gram, mm: millimeter, SOH: sweetened alcohol, NA: Naringin; SOH+NA: Sweetened alcohol and naringin

6.4 Discussion

In this chapter, I explored the effects of naringin and sweetened alcohol on erythrocyte indices, cardiac troponin-I, GIT, and accessory organs' morphometry. Sweetened alcohol consumption and naringin supplementation had no effects on all the parameters studied. However, looking at the organ's morphometry, it was observed that male rats had higher absolute mass, while female rats had increased organ mass indexed to body mass.

Although they do so in different ways, alcohol and fructose can both have a major effect on erythrocyte indices.

Membrane fluidity and deformability: Alcohols can change the mechanical characteristics and membrane fluidity of erythrocytes. According to studies, depending on the concentration, alcohols such as ethanol can first increase and then decrease the deformability of red blood cells (Sonmez *et al*., 2013). This biphasic effect is linked to changes in membrane fluidity.

Haematological abnormalities: Chronic alcohol consumption can lead to various haematological abnormalities, including macrocytosis, anaemia, and thrombocytopenia (low platelet count). These effects are due to both direct toxic effects on bone marrow and indirect effects, such as nutritional deficiencies (Saka *et al.,* 2011; Yakubu *et al.,* 2007).

Oxidative stress: High fructose intake can induce oxidative stress in erythrocytes, leading to damage of the cell membrane and haemoglobin. This can result in decreased cell lifespan and increased hemolysis (Pazzini *et al*., 2015).

Metabolic effects: Byproducts of the liver's metabolism of fructose may have an impact on erythrocyte function. For instance, too much fructose can cause advanced glycation

end-products (AGEs), which can increase red cells' rigidity and affect their function (Gugliucci, 2017).

These mechanisms show that alcohol and fructose can affect erythrocytes' function negatively. However, the intervention used in this study did not affect the rats' red cell indices. This shows that the concentrations of alcohol and fructose used have no effect on the rats' red blood cell's function.

To support the findings from this study, in another study where rats were fed alcohol and fructose, there were no significant differences in erythrocyte indices compared with the control (Alwahsh *et al.,* 2014). There were also no differences in Hb and PCV values in animals consuming alcohol (Lugos *et al.,* 2018) compared to the control.

The findings on cardiac troponin-I align with the findings in chapter three that show neither sweetened alcohol nor naringin were associated with significant alteration in cardiac function. However, other studies have reported raised levels of cardiac troponin-I following fructose (Adoga *et al.,* 2021), and alcohol consumption (Csengeri *et al*., 2021), which is contrary to the findings in our study. The amount of alcohol used in Csengeri's (2021) study was said to have caused intoxication in the rats, which might induce cardiac injury and lead to increased levels of cardiac troponin-I in the study. The concentration of alcohol and the duration of consumption might explain the reason for the lack of effects on cardiac troponin-I by the intervention in this study.

I observed no group differences in absolute and relative masses of visceral organs in male and female rats. However, the study observed that male rats had increased absolute masses of the visceral organs and increased absolute length of the small and

large intestines, but the increase was greater in females when the measurements were indexed to body mass.

This phenomenon can be attributed to several factors related to biological and physiological differences between males and females:

Absolute Mass vs. Indexed Mass

1. Absolute mass: In general, males tend to have larger absolute organ masses due to their overall larger body size and higher muscle mass. This is influenced by higher levels of testosterone, which promote muscle and bone growth (West *et al*., 2010).
2. Indexed mass: When organ mass is indexed to body mass (i.e., organ mass divided by total body mass), some organs may appear heavier in females. This is because females typically have a higher percentage of body fat and lower muscle mass compared to males (Bredella, 2017). As a result, when adjusting for body mass, the relative size of certain organs can be proportionally larger in females.
3. Hormonal influences: Hormones like estrogen and progesterone in females, and testosterone in males, play significant roles in organ development and function. These hormonal differences contribute to variations in organ size and function between the sexes (Bhargava *et al*., 2021).

As seen from this study, other studies also reported that alcohol consumption did not affect the weight of the testes (Costa-Valle *et al*., 2022), while fructose consumption has been linked to reduced testicular mass in rats (Shibata & Fukuwatari, 2013). However, the fructose used in their study was higher (80%) than the fructose used in

this study (20%), which explains the disparity. The increased epididymal fat mass has been associated with increased inflammatory changes, which can lead to inflammation of the epididymis, causing reproductive dysfunction (Jia *et al*., 2018). The findings on epididymal fat seen in male rats align with those reported in the previous chapters, which reported no differences in body mass and visceral fat mass in male rats, as epididymal fat is seen as part of the abdominal fat, even though the testes and epididymis are not part of the visceral organs (Item & Konrad, 2012). Naringin supplementation did not affect the epididymal fat mass, which emphasised the findings on body mass and visceral adiposity in males as reported in the previous chapters.

6.5 Conclusion

The voluntary consumption of sweetened alcohol and naringin had no effects on erythrocyte indices, GIT morphometry and Troponin-I in male and female Sprague-Dawley rats. This suggests that the model used did not result in overt toxicity to the tissues.

The results of this study are summarised, some limitations are noted, and suggestions for further research are made in the next chapter.

CHAPTER SEVEN - OVERALL CONCLUSIONS, LIMITATIONS, RECOMMENDATIONS, AND FUTURE DIRECTIONS

7.1 Summary and conclusion

Sweetened alcohol consumption is on the rise, particularly among adolescents and young adults, and because of its palatability and increased accessibility and availability, it may result in a double burden of increased sugar and alcohol intake. Its possible effects on cardiometabolic disorders are still unclear. This study evaluated the potential therapeutic and preventive effects of oral naringin as a dietary supplement to adolescent male and female Sprague-Dawley rats on the development of cardiometabolic derangements induced by voluntary consumption of sweetened alcohol. Four major areas were specifically focused on, including:

1. Cardiac dysfunction (evaluated by echocardiographic and blood pressure measurements, heart mass, and histology of the left ventricle).
2. Metabolic dysfunction, hepatic steatosis, and visceral obesity - MAFLD (by evaluating/measuring feed and water intake, gelatine consumption, body mass, FBG, serum TG, insulin, visceral fat mass, epididymal fat pad (males), liver mass, and liver histology).
3. Nephropathy (by evaluating/measuring kidney mass, serum NGAL, serum KIM-1, and kidney histomorphometry)
4. General cardiometabolic health derangements (by evaluating/measuring erythrocyte indices – PCV, Hb, MCHC, cardiac troponin-I, morphometry of the small and large intestine, stomach, caecum, pancreas, and testes- for males)

Sweetened alcohol caused concentric cardiac remodelling, decreased left ventricular relaxation, and increased filling pressures in females (features of systolic and diastolic dysfunctions), and hepatic steatosis (fatty liver) in both male and female rats. In male rats, sweetened alcohol led to decreased serum NGAL, KIM-1, and glomerular urinary space. In contrast, in female rats, it led to decreased serum NGAL, increased KIM-1

concentration, and increased glomerular density (early features of nephropathy). The study shows that female rats are more prone to sweetened alcohol-induced cardiometabolic derangements.

Naringin supplementation improved concentric cardiac remodelling, prevented hepatic steatosis, and decreased serum TG, serum NGAL, and KIM-1 in females, while in males, naringin caused decreased serum NGAL and increased serum KIM-1 concentrations. When supplemented with sweetened alcohol, naringin prevented hepatic steatosis and led to decreased serum insulin, increased FBG, increased glomerular density, and increased serum KIM-1 in females, while in males, it led to increased serum KIM-1 and NGAL concentrations. The mechanisms through which naringin improved these cardiometabolic indices may be related to other studies that reported its anti-inflammatory and antioxidant (Shilpa *et al.,* 2023), cardioprotective (Uryash *et al.,* 2021), hepatoprotective (Jutrić *et al.,* 2022), nephroprotective (Amini *et al.,* 2022) effects, and the ability to improve other metabolic dysfunctions (Kumar *et al*., 2019).

In conclusion, with the increased consumption and popularity of sweetened alcohol and its relation to increased alcohol consumption, the risk of cardiometabolic dysfunction is imminent. Strategic intervention to prevent these dysfunctions may be of great benefit to public health. Strategic intervention with phytochemicals such as naringin in the form of supplements in terms of its dose, mechanisms of action, gender differences, and possibly its side/adverse effects needs to be further explored. However, supplementing naringin with sweetened alcohol should be done with caution as it may negatively affect the kidneys and some metabolic functions, as seen from this study. Limitations and future directions

The study looked at the combined effects of alcohol and fructose in the form of sweetened alcohol. However, the study did not look at the individual effects of alcohol and fructose incorporated into gelatine. Individual effects of alcohol and fructose incorporated into gelatine may have provided a better comparison of the individual and combined effects. Also, a different range of doses of the interventions (alcohol, fructose, and naringin) might have helped to determine dose-response relationships for all the interventions. For the naringin, if different ranges of doses were used, it would have provided us with a safer dose to treat and/or prevent sweetened alcohol-induced cardiometabolic derangements, especially for the kidney. I therefore recommend future studies to consider these.

Cardiomyocyte diameter was assessed as one of the indices of cardiac function using H&E staining. Even though it has provided information on the histopathological changes in the left ventricular tissue, however, Gomori's trichrome and wheat germ agglutinin (WGA) stains give superior specificity and quantitative precision for evaluating cardiomyocyte size. Therefore, using these stains would have provided additional insights into the evaluation of cardiac remodelling and should therefore be considered in future studies. Echocardiography has been used in this study to assess cardiac function. However, using electrocardiography may have also provided additional information about the cardiac function. This should also be considered in future studies.

In this study, sweetened alcohol induced visceral obesity and hepatic steatosis. These are associated with oxidative stress and inflammation (Fricker *et al*., 2019; Klisic *et al*., 2018). These findings can be strengthened by measuring antioxidants such as Thiobarbituric acid reactive substances (TBARS), Malondialdehyde (MDA), Superoxide dismutase (SOD), and anti-inflammatory mediators such as interleukin-6

(IL-6), tumour necrosis factor-alpha (TNF-α), and C-reactive protein (CRP). I therefore recommend that future studies should consider exploring the measurement of these biomarkers. Other indicators of liver function, such as aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transferase, were also not measured in this study. They are also vital in assessing liver function. I also recommend measuring these markers in future studies. The molecular mechanisms of sweetened alcohol-induced hepatic steatosis were not explored in this study; I therefore recommend future studies to consider investigating the molecular mechanisms of sweetened alcohol-induced hepatic steatosis. In assessing renal function, this study did not measure the traditional laboratory indicators of renal function such as serum/urinary creatinine, urea, estimated glomerular filtration rate (eGFR), and urinary albumin. Measuring these markers together with NGAL and KIM-1 might provide additional diagnostic value relative to sweetened alcohol and/or naringin with kidney injury. It may also help to classify whether the kidney insult is an acute or chronic one. I, therefore, recommend that future studies should consider measuring these markers concurrently. In addition, even though H&E stain was able to show some histopathological changes of the kidney, however, other stains such as Mason's Trichrome stain and Periodic Acid- Schiff (PAS) would have provided more details on kidney tissue fibrosis and specific tubular secretions, thus providing additional insight into the potential mechanisms behind any pathology in the kidney. In the future, the use of electron microscopy and immunohistochemistry studies will shed more light on the early changes in kidney architecture.

Molecular studies associated with naringin effects on sweetened alcohol-induced cardiometabolic dysfunctions have not been carried out in this study. However, naringin has been reported to exert improved cardiometabolic effects through its anti-inflammatory and antioxidant effects and by modulating and regulating different

metabolic pathways (Peng *et al.,* 2024). I therefore recommend molecular studies in future to evaluate the molecular mechanism(s) associated with the effects of naringin associated with sweetened alcohol-induced cardiometabolic dysfunction. Also, molecular mechanisms through which naringin supplemented with sweetened alcohol negatively affected some kidney and metabolic functions need to be carefully studied in the future.

CHAPTER EIGHT - REFERENCES

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APPENDICES

APPENDIX 1: PLAGIARISM DECLARATION

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I JELANI MUHAMMAD (Student number: 1332401) am a student registered for the degree of PhD in the academic year 2025.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature:  Date: 28-03-2025

1

APPENDIX 2: ETHICAL CLEARANCE

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/10/02/C

APPLICANT: Dr J Muhammad
School: School of Physiology; Department: ; Location: WRAF

PROJECT TITLE: Potential of Naringin to prevent sweetened alcohol-induced cardiometabolic derangements in adolescent Sprague-Dawley rats
Category: C; Species and Numbers involved: 80X 42 days old, male or female, Sprague-Dawley Rats

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 25 Oct 2022. This approval remains valid until 14 Dec 2024 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ _____ Date: ___10/01/2023_____

(Chairperson of the AREC)

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

Signed: ___ _____ Date: _13 /01/2023_____

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

APPENDIX 3: FIRST MODIFICATION AND EXTENSION OF THE ETHICS CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

AREC 2022 AM&E

Please note that only electronically completed applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
ANIMAL RESEARCH ETHICS COMMITTEE

registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, reg. no. AREC -101210-002

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Principal Investigator (PI) Name: MUHAMMAD JELANI

b. School / Department: School of Physiology

c. Email Address: 1332401@students.wits.ac.za

d. Experiment to be modified / extended **AREC NO**

Original AREC Clearance Certificate Number	2022	10	02/ C
Clearance Certificate Expiry date	14 DECEMBER 2024		
Number of previous AM&Es			0

e. Project Title: **Potential of Naringin to prevent sweetened alcohol-induced cardiometabolic derangements in adolescent Sprague-Dawley rats**

		No.	Species
f.	Number and species of animals originally approved	80	Sprague Dawley Rats
g.	Number of additional animals previously approved on AM&Es	None	
i.	Number of animals used and currently involved in the study to date	80	

j. Specific details of Adaptations/Modification/ Extension:

Extension of ethics clearance certificate expiry date

k. Motivation for Adaptations/Modification/ Extension:
The laboratory analysis for the study is ongoing and will extend beyond the clearance certificate expiry date.

Date: 08 November, 2024 PI Signature: 

RECOMMENDATIONS:
Approve the extension of study to 30 December 2026.

Date: 20 November 2024 Signature: 

Chairperson

APPENDIX 4: SECOND MODIFICATION AND EXTENSION OF THE ETHICS CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

AREC 2022 AM&E

Please note that only electronically completed applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
ANIMAL RESEARCH ETHICS COMMITTEE

registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, reg. no. AREC -101210-002

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Principal Investigator (PI) Name: MUHAMMAD JELANI

b. School / Department: School of Physiology

c. Email Address: 1332401@students.wits.ac.za

d. Experiment to be modified / extended **AREC NO**

Original AREC Clearance Certificate Number	2022	10	02/ C
Clearance Certificate Expiry date	30 DECEMBER 2026		
Number of previous AM&Es			1

e. Project Title: **Potential of Naringin to prevent sweetened alcohol-induced cardiometabolic derangements in adolescent Sprague-Dawley rats**

		No.	Species
f.	Number and species of animals originally approved	80	Sprague Dawley Rats
g.	Number of additional animals previously approved on AM&Es	None	
I.	Number of animals used and currently involved in the study to date	80	

j. Specific details of Adaptations/Modification/ Extension:

To add co-workers

i. Mr Booki Ntai (student number 2442974@students.wits.ac.za) from the department of Physiology

ii. Miss Jyoti Patel (student number 2362006@wits.ac.za) from the department of Physiology

iii. Dr Ashmeetha Manilall from the department of Physiology

k. Motivation for Adaptations/Modification/ Extension:

i. Mr Ntai has joined the department of Physiology as an Honours student. We would like to include him as a co-worker on the study for purposes of the research report for his degree. He will investigate the impact of the interventions on histological structure of triceps muscle samples using histology and will assay serum samples (using ELISA) for markers of muscle damage (skeletal troponin I, myosin light chain 3, fatty acid binding protein 3, and Creatine Kinase)

ii. Miss Jyoti has joined the department of Physiology as an Honours student. We would like to include her as a co-worker on the study for purposes of the research report for her degree. She will investigate the impact of the interventions on histological structure of small intestine using microscopy and will assay serum samples (using ELISA) for markers of intestinal permeability (Flagellin, Lipopolysaccharide and Il-6).

iii. Dr Ashmeetha Manilall (ashmeetha.manilall@wits.ac.za), will serve as a co-supervisor on

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the projects and provide technical assistance.

Date: 03 March, 2025

PI Signature: 

RECOMMENDATIONS:
Approve the addition of the above-mentioned co-workers to the study.

Date: 04 March 2025

Signature: 

Chairperson

APPENDIX 4: PUBLISHED MANUSCRIPT

Received: 13 March 2024 | Revised: 19 August 2024 | Accepted: 23 August 2024

DOI: 10.14814/phy2.70030

ORIGINAL ARTICLE

PHYSIOLOGICAL REPORTS

Effects of voluntarily consumed sweetened alcohol and naringin on cardiac function in male and female Sprague–Dawley rats

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Abstract

This study assessed the impact of sweetened alcohol and naringin on cardiac function in Sprague-Dawley rats. Male ($n=40$) and female ($n=40$) rats were allocated to control, sweetened alcohol (SOH), naringin (NA), and sweetened alcohol with naringin (SOH + NA) groups. SOH and SOH + NA rats received 10% alcohol + 20% fructose in gelatine; SOH + NA and NA rats received 50 mg/kg naringin in gelatine daily for 10 weeks. Echocardiography was performed to assess left ventricular (LV) function. LV cardiomyocyte diameters and collagen area fraction were determined by H&E and picrosirius-red staining, respectively. In males, sweetened alcohol and naringin did not affect cardiac function. Female SOH rats had increased LV end-diastolic posterior wall ($p=0.04$), relative wall thicknesses ($p=0.01$), and LV cardiomyocyte diameters ($p=0.005$) compared with control. Female SOH and SOH + NA had reduced lateral e' and e'/a' and increased E/e' ($p<0.0001$). Female SOH ($p=0.01$) and SOH + NA ($p=0.04$) rats had increased LV collagen area fraction compared with controls. In males, neither sweetened alcohol nor naringin affected cardiac geometry or diastolic function. In females, sweetened alcohol induced concentric remodelling, impaired LV relaxation, and elevated filling pressures. Naringin may have the potential to improve the sweetened alcohol-induced concentric remodelling; however, it did not ameliorate diastolic dysfunction in females.

KEYWORDS
cardiac geometry, diastolic function, naringin, sweetened alcohol

1 | INTRODUCTION

Modern society has seen a rise in the popularity of sweetened alcoholic beverages, including flavored spirits, jello shots, and cocktails (Crews, 2015; Mosher & Johnsson, 2005; Wakabayashi et al., 2021). The sweeteners incorporated into these beverages mask the presence of alcohol, making the beverages more palatable and consequently less apparent how much alcohol is being ingested. This can increase the risk of overconsumption, particularly among adolescents who often start drinking these beverages at a young age (Binakonsky et al., 2011; Crews, 2015; Mola et al., 2023). Common sweeteners in these beverages typically include sucrose (a blend of 50% glucose and 50% fructose), high fructose corn syrup, or concentrates from fruit juice (primarily fructose) (Poppitt, 2015).

The combination of alcohol and fructose in these drinks poses a double health risk burden as individually, alcohol and fructose have been associated with negative cardiometabolic health outcomes such as obesity, dyslipidaemia, and insulin resistance (Poppitt, 2015). These modifiable risk factors may lead to the development of cardiovascular diseases such as heart failure and atherosclerosis (Liu et al., 2022; Meijers & De Boer, 2019; Tan et al., 2020). A high fructose diet may cause cardiac inflammation and fibrosis through various mechanisms including increased production of pro-inflammatory factors, upregulation of pro-fibrotic gene expression, macrophage infiltration, and heightened oxidative stress (Kang et al., 2015; Wang et al., 2023; Xu et al., 2022; Zhang et al., 2016). Alcohol consumption may result in increased reactive oxygen species (ROS) leading to reduced endothelial nitric oxide synthase in cardiomyocytes (Tsermpini et al., 2022; Wang et al., 2015). Therefore, the combined effects of fructose and alcohol may cause several structural changes as well as functional disturbances in the heart which may predispose individuals to cardiac dysfunction. Although fructose and alcohol individually have been extensively studied for their effect on cardiac function, their combined effects remain an area of ongoing investigation.

Managing the cardiometabolic consequences of alcohol and high fructose consumption results in a heavy socioeconomic burden for many countries globally (Rao, 2018). There is a need to explore prophylactic interventions early in life to reduce the poor health outcomes associated with alcohol and sugar consumption. Phytochemicals are increasingly being targeted as candidates for nutraceutical potential (Bruno et al., 2022).

Naringin, a flavonoid phytochemical found in citrus fruit, has recently become of interest for its possible cardioprotective effects (Akin et al., 2022; Malakul et al., 2018; Park et al., 2018; Shackebaei et al., 2023; Uryash et al., 2021). Studies have reported that naringin has antioxidant and anti-inflammatory properties (Akin et al., 2022; Uryash et al., 2021) that may protect the myocardium by reducing inflammation as well as oxidative stress that takes place after the consumption of fructose or alcohol. Nevertheless, to the best of our knowledge, there is no literature regarding the effects of naringin on sweetened alcohol-induced cardiac changes. Consequently, further studies are required to determine naringin's potential cardioprotective effects in the context of sweetened alcoholic beverage consumption. There is a bias towards the use of males in animal-based studies despite the societal trends showing that both males and females consume sweetened alcoholic beverages (Wakabayashi et al., 2021). Therefore, this research aimed to investigate the effects of sweetened alcohol and naringin on cardiac function in male and female Sprague–Dawley rats.

2 | METHODS

2.1 | Ethical clearance and study site

The study adhered to international ethical guidelines and standards governing the care and utilization of laboratory animals, receiving approval from the Animal Research Ethics Committee of the University of Witwatersrand (AREC clearance certificate number 2022/10/02/C). The research took place at the Wits Research Animal Facility (WRAF) situated at the University of the Witwatersrand in Johannesburg, South Africa. The study adhered to the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines both during the research process and in the compilation of the manuscript.

2.2 | Animals and study design

A total of 80, 42-day-old Sprague–Dawley rats (comprising 40 males and 40 females), were sourced from the WRAF of the University of the Witwatersrand, Johannesburg. Each rat was housed in a perspex cage topped with steel mesh covers. The cages were lined with wood shavings and shredded paper for bedding and environmental enrichment.

Additional environmental enrichment included wooden blocks for the rats to gnaw on and sections of wide bore pipes for the rats to hide in. Rats were provided with ad libitum access to standard pelletized rat chow (LabChef®, Johannesburg, South Africa) and tap water for the entirety of the study. To maintain a hygienic environment, cages, and bedding were changed twice a week. The room's ambient temperature was controlled at 26 ± 2°C, with appropriate ventilation and a 12-h light/dark cycle (lights on at 07.00 h).

The experiment was designed as a randomized, interventional prospective study. The rats were subjected to a 1-week acclimatization period wherein initial assessments, including body mass, food consumption, and blood pressure were recorded. The rats were also acclimatized with gelatine which was prepared by adding 0.5 g fructose with 6 g gelatine in 100 mL of water. It was given daily at 10 mL/100 g body weight for all the rats during the acclimatization period. Following this acclimatization period, rats were randomly assigned to one of four treatment groups, each consisting of 20 rats (10 males and 10 females):

1. Control: received plain gelatine (0.5% fructose in 6% gelatine)—fructose was added to the gelatine to improve the palatability of the preparation.
2. Sweetened alcohol (SOH): received sweetened alcohol (10% alcohol +20% fructose) in 6% gelatine.
3. Naringin (NA): received 50 mg/kg body mass (BM) naringin in gelatine (0.5% fructose in 6% gelatine).
4. Sweetened alcohol + naringin (SOH + NA): received sweetened alcohol (10% alcohol +20% fructose) + 50 mg/kg BM naringin in 6% gelatine.

The gelatine with or without alcohol and/or naringin was provided daily to the rats in glass containers at a volume of 10 mL per 100 g body mass. The use of gelatine for voluntary administration of alcohol has been previously described and reported in various studies (Dess et al., 2013; Peris et al., 2006; Ralevski et al., 2006).

Before the commencement of the intervention, baseline blood pressure and body masses were measured. The study design is depicted in Figure 1 below.

2.3 | Interventions used and preparation

For our study, gelatine (Sheridan's gelatine, Libstar Operations (Pty) Ltd, Dunkeld, South Africa) was used as a vehicle. Plain gelatine was prepared by mixing 6 g gelatine with 0.5 g fructose powder (Fructose, Nature's Choice, Randvaal, South Africa) and 1–2 mL Bovril (Beefy Bovril, Pioneer Foods (Pty) Ltd, South Africa) in 100 mL of hot water. Naringin was incorporated into gelatine by

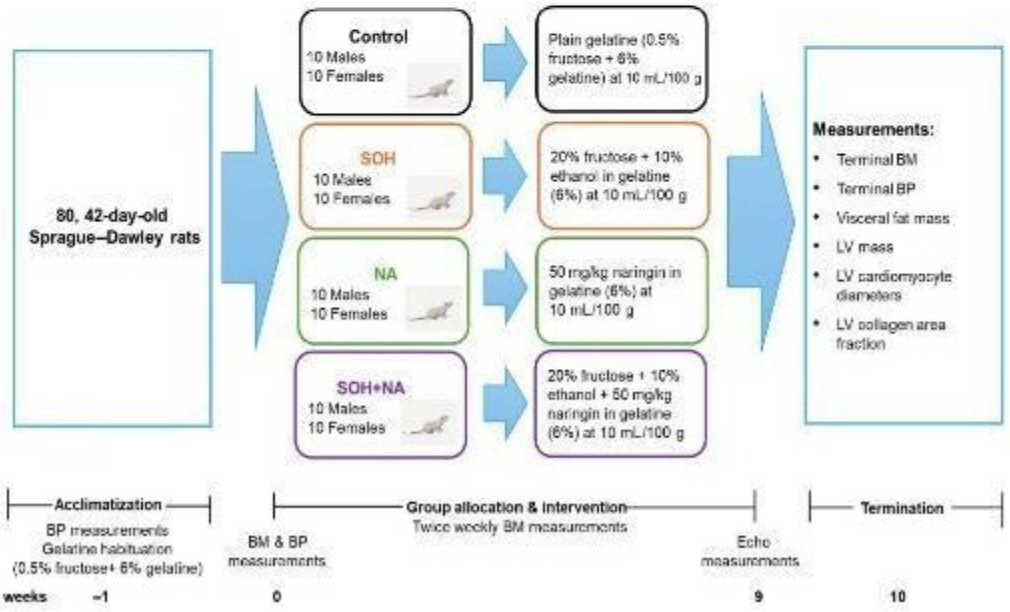

FIGURE 1 Schematic diagram of the study design. BP, blood pressure; BM, body mass; Echo, echocardiography; LV, left ventricle; NA, naringin; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

adding 0.05 g (to provide a dose of 50 mg/kg body mass (Adebiyi et al., 2016)) naringin (>95% HPLC, CAS No. 10236-47-2, Sigma-Aldrich, China) to the plain gelatine as described above. Sweetened alcohol in gelatine was prepared by adding 20 g fructose powder (equivalent to 20% fructose (Komnenov et al., 2020)), 6 g gelatine, and 1–2 mL Bovril, with 10 mL of ethanol (equivalent to 10% ethanol (Al-Awwadi et al., 2004)) (Ethanol 99.9%; CAS No. 64-17-5; Batch No. 37500; ACE chemicals, South Africa) to provide up to a maximum ethanol dose of 0.79 g/100 g body weight, in 90 mL of hot water (<60°C). This dose is equivalent to about half of a glass/unit of alcohol in humans which corresponds to chronic light alcohol consumption (Brick, 2006) and has been associated with elevated liver enzymes (Niemelä et al., 2017). Sweetened alcohol with naringin was prepared by adding 0.05 g naringin (to provide a dose of 50 mg/kg body mass) of naringin into the sweetened alcohol in gelatine preparation as described above (doses were adjusted to the rats' body mass). In each case, the mixture was thoroughly mixed using a magnetic hot plate stirrer. The solutions/mixtures were then placed into glass containers and sealed to prevent evaporation of the alcohol. All preparations were kept and allowed to set in a fridge at 2–7°C for 4–24 h before being served to the rats. All treatments (either plain gelatine, sweetened alcohol, naringin in gelatine, or sweetened alcohol with naringin) were offered to the rats once daily (at approximately 2:00 PM) at a dosage of 10 mL per 100 g body mass over 10 weeks. The gelatine preparation consumed by each rat was estimated by subtracting the remaining gelatine in milliliters (mL) from the gelatine provided each week. Relative gelatine consumption was determined by dividing the gelatine consumed by body mass and then multiplying by 100.

2.4 | Body mass measurements

An electronic weighing balance (Snowrex EQ-1200, Snowrex International Company, Taipei, Taiwan) was used to measure the baseline body mass. Subsequently, measurements were conducted twice weekly to assess growth progress and make necessary adjustments to the dosage of fructose, gelatine, ethanol, and naringin, thereby ensuring a consistent dosage proportionate to the body mass. The final body mass was measured at termination.

2.5 | Noninvasive blood pressure measurements

Blood pressure was measured at baseline and termination using the tail-cuff technique (Biopac Systems, Santa Barbara, CA, USA), as previously described (Pauline et al., 2011). Briefly, each rat (priorly habituated to the procedure) was put in a restrainer, with the rat's tail on a heating pad. After the rats' tails were heated, the blood pressure machine's cuff was placed at the base of the tail, and readings of blood pressure were recorded. Two to three readings per rat were taken and the average was determined. To prevent diurnal variation, measurements were done at midday.

2.6 | Echocardiography measurements

Nine weeks after the commencement of the intervention, rats were anesthetized with isoflurane (1%–3% in oxygen). Anesthesia was first induced in a chamber and then maintained with a face mask. Following this, the anterior chest wall was shaved.

Echocardiography was performed with the rats in the left lateral decubitus position using a high-resolution ultrasound probe (10 MHz) coupled to an echocardiographic machine (Affiniti CVx, Philips Healthcare, Andover, Massachusetts). LV dimensions were determined using two-dimensional directed M-mode echocardiography in the parasternal long-axis view during three consecutive beats. LV end-systolic and end-diastolic internal diameters, interventricular septal thickness, and posterior wall thickness were measured in systole and diastole. Relative wall thickness was calculated as (2 × posterior wall thickness)/(LV internal diameter at end-diastole) (Lang et al., 2015). LV pump and myocardial systolic function were determined by calculating LV ejection fraction using the Teichholz method and LV endocardial fractional shortening (Lang et al., 2015).

LV diastolic function was determined from the mitral valve inflow patterns using pulsed Doppler imaging. In the apical 4-chamber view, the early (E) and late (A) diastolic inflow velocities were obtained with the sample volume placed at the mitral valve leaflet tip and expressed as E/A, a marker of relaxation. To determine diastolic function using tissue Doppler imaging (TDI), peak myocardial tissue lengthening velocities during early (e') and late (a') diastole were recorded at the lateral mitral annulus in the apical four-chamber view. Diastolic function markers are expressed as e' (an index of myocardial relaxation), e'/a' (an index of myocardial stiffness), and E/e' (an index of LV filling pressure).

The rats were allowed to recover from the anesthetic and then returned to their cages and respective treatments for a week.

Measurements were taken in the LV since the LV plays a crucial role in ensuring blood circulates to all parts of the body. Given its thicker wall compared to the right

ventricle, the LV is more often susceptible to various cardiovascular conditions, and therefore, studying the left side of the heart provides meaningful information into the overall cardiac function (Kerfoot et al., 2019). Moreover, cardiovascular diseases such as heart failure, myocardial infarction, and hypertension, predominantly affect the left side of the heart (Tsao et al., 2015). Therefore, focusing on the left side provides a better understanding of the mechanisms associated with these conditions.

2.7 | Terminal procedures

At the end of the 10-week intervention, rats were euthanized using a sodium pentobarbital overdose (Euthapent, Kyron laboratories, Johannesburg, South Africa) at 150 mg/kg BM injected intraperitoneally. Visceral fat (adjoining the liver, kidneys, pancreas, stomach, and small and large intestines) was carefully removed, and weights were determined using a Presica 310M digital scale (Precision Instruments, Johannesburg, South Africa). The hearts were dissected out and their respective weights were recorded. Subsequently, the left ventricle was isolated from the other cardiac structures, and its specific weight was determined using a Presica 310 M digital scale (Precision Instruments, Johannesburg, South Africa).

2.8 | LV cardiomyocyte diameter and total collagen content determination

LV tissue was preserved in 10% phosphate-buffered formalin and later histologically examined. For the histological examination, the samples were processed using the Thermo Scientific's Microm STP 120 (Microm STP 120, Thermo Scientific, MA, USA) automatic tissue processor, and embedded in paraffin wax. Processed tissues were sectioned at 4 µm thick using a microtome (Leica Biosystems Instruments (Pty), Ltd, model RM 2125, Wetzlar, Germany) and placed onto glass microscope slides. The slides were allowed to air-dry and adhere to a 20°C heating block.

2.8.1 | Hematoxylin and eosin stain

The Hematoxylin and Eosin (H&E) stain was used to assess LV cardiomyocyte diameter (width) at the level of the nucleus in longitudinal sections. Briefly, the samples underwent deparaffinization with xylene, rehydration through a series of graded ethanol concentrations, and staining with Harris' hematoxylin followed by alcoholic eosin solution. Subsequently, the slides were washed (dehydrated) with ethanol and xylene before being mounted with DPX mountant and covered with a coverslip. The slides were visually analyzed by a blinded evaluator using a Zeiss Axioskop 2 Plus microscope, equipped with a Zeiss AxioCam (Zeiss, Peabody, MA, USA), to analyze the LV cardiomyocyte diameter. Tissue sections viewed under a bright field were obtained using a 4× objective lens (×400 magnification). The LV cardiomyocyte diameters at the level of the nucleus in longitudinal sections were determined using the straight-line tool on ImageJ software (ImageJ version 1.54d, Laboratory for Optical and Computational Instrumentation, University of Wisconsin) (Baudouy et al., 2017). LV cardiomyocyte diameters were measured three times per sample and an average was determined.

2.8.2 | Picrosirius red stain

The picrosirius red stain was used to measure the accumulation of collagen fibers to quantify the degree of fibrosis in LV samples. Briefly, sections were deparaffinized, rehydrated, and stained with a 0.1% Sirius Red solution dissolved in aqueous saturated picric acid for 60 min at room temperature. After washing in acidified water (5 mL of acetic acid into 1 L of distilled water), the slides were dehydrated and mounted with DPX mountant (Merck Life Science (Pty) Ltd, Modderfontein, South Africa). The tissue sections were visually analyzed by a blinded evaluator using a Zeiss Axioskop 2 Plus microscope equipped with a Zeiss AxioCam (Zeiss, Peabody, MA, USA). Tissue section images viewed under bright-field and polarized light were obtained using a 10× objective lens (x100 magnification). The collagen area fraction and overall tissue area for each tissue section were determined using ImageJ software (ImageJ version 1.54d, Laboratory for Optical and Computational Instrumentation, University of Wisconsin). Collagen area fractions were determined three times per sample and an average was determined. The collagen area fraction was computed by dividing the collagen area by the overall tissue area (Baidoo et al., 2022).

2.9 | Statistical analysis

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc. Cary, North Carolina, USA). Graphs were generated using GraphPad Prism version 9.4 (GraphPad Software Inc., San Diego, CA, USA).

The sample size was determined using the formula (Charan & Kantharia, 2013): $2\ SD^2\ (1.96+0.842)^2/d^2$ where SD = standard deviation; 1.96 = type 1 error of 5%; 0.842 = at 80% power; 0.842 is a value from the standard formula used to determine the sample size. The value (0.842) is associated with the Z-score, or critical value related to

achieving 80% power in a standard normal distribution. When using a standard normal distribution, a Z-score of approximately 0.842 corresponds to 80% power (Charan & Kantharia, 2013), and d = difference between mean values.

Shapiro–Wilk tests were used for normality tests. Data were normally distributed and are expressed as means ± SEM. To determine differences in baseline and terminal body masses and blood pressure measurements, as well as weekly gelatine consumption measures, repeated measures two-way analysis of variance (ANOVA) was used with group and sex as the main effects. If significant differences were observed, Tukey post hoc tests were employed to identify specific group and time differences. Cardiac geometry and functional differences were assessed using two-way ANOVA with group and sex as the main effects. If significant differences were observed, Tukey post hoc tests were employed to identify specific group differences. Associations between tissue Doppler indices and the collagen area fraction were determined using Pearson's correlation. Since blood pressure contributes to the development of diastolic dysfunction, blood pressure was included as a confounder in the correlation analysis. $p < 0.05$ was considered as significant.

3 | RESULTS

3.1 | Morbidity and mortality

One of the male rats died from the Male SOH + NA group during the study due to iatrogenic causes (confirmed by autopsy).

3.2 | Body mass, visceral fat mass, and blood pressure measurements

Table 1 shows the effects of sweetened alcohol and naringin on body mass and blood pressure at baseline and at termination in male and female Sprague–Dawley rats. The group and sex effects were significantly different for baseline body mass (group: $F = 2.49$, $p = 0.02$; sex: $F = 13.27$, $p = 0.0005$) and terminal body mass (group: $F = 175.60$, $p < 0.0001$; sex: $F = 1208.68$, $p < 0.0001$). Body masses were significantly higher ($p < 0.0001$) at termination compared to baseline across all groups in both male and female rats. Males were heavier ($p < 0.0001$) compared to females at baseline and termination.

The group and sex effects were significantly different for visceral fat mass indexed to body mass (group: $F = 16.44$, $p < 0.0001$; sex: $F = 76.13$, $p < 0.0001$). Female SOH and SOH + NA rats had heavier visceral fat masses indexed to body mass compared to female control and NA rats ($p < 0.0001$). Female SOH and SOH + NA rats had heavier visceral fat masses indexed to body mass compared to all male groups ($p < 0.0001$).

There were no significant differences observed in group and sex effects for baseline systolic blood pressure (group: $F = 0.42$, $p = 0.89$; sex: $F = 0.13$, $p = 0.72$) and terminal systolic blood pressure (group: $F = 0.81$, $p = 0.58$; sex: $F = 0.11$, $p = 0.74$). There were no significant differences observed in group and sex effects for baseline diastolic blood pressure (group: $F = 1.13$, $p = 0.31$; sex: $F = 0.02$, $p = 0.92$) and terminal diastolic blood pressure (group: $F = 1.15$, $p = 0.34$; sex: $F = 0.00$, $p = 0.96$).

TABLE 1 Effects of sweetened alcohol and naringin on body mass, visceral fat mass, and blood pressure at baseline and termination in male and female Sprague–Dawley rats.

	Males				Females			
	Control	SOH	NA	SOH + NA	Control	SOH	NA	SOH + NA
Baseline								
Body mass (g)	148 ± 6[δ]	144 ± 6[δ]	154 ± 6[δ]	156 ± 6[δ]	118 ± 6[δ,α]	122 ± 6[δ,α]	111 ± 6[δ,α]	132 ± 6[δ,α]
SBP (mm Hg)	116 ± 4	119 ± 2	124 ± 3	118 ± 4	119 ± 3	120 ± 3	120 ± 3	122 ± 3
DBP (mm Hg)	79 ± 2	75 ± 3	72 ± 2	76 ± 3	75 ± 3	78 ± 3	75 ± 3	79 ± 2
Termination								
Body mass (g)	463 ± 6	435 ± 6	460 ± 6	428 ± 6	258 ± 6[α]	271 ± 6[α]	257 ± 6[α]	268 ± 6[α]
Visceral fat (% body mass)	2.79 ± 0.38	2.74 ± 0.38	2.87 ± 0.41	2.87 ± 0.41	3.97 ± 0.38	6.17 ± 0.38[*,α]	4.09 ± 0.38	6.64 ± 0.38[*,α]
SBP (mm Hg)	117 ± 1	115 ± 3	119 ± 3	117 ± 2	119 ± 3	117 ± 2	117 ± 2	119 ± 3
DBP (mm Hg)	79 ± 2	79 ± 2	76 ± 2	78 ± 3	78 ± 2	79 ± 1	81 ± 1	77 ± 1

Note: Data expressed as mean ± SEM. *$p < 0.05$ compared to control. [α]$p < 0.05$ compared to NA. [δ]$p < 0.0001$ compared to terminal body mass. [α]$p < 0.0001$ compared to all male groups.

Abbreviations: DBP, diastolic blood pressure; NA, naringin; SBP, systolic blood pressure; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

3.3 | Gelatine consumption

Figure 2 shows the effects of sweetened alcohol and naringin on weekly absolute and relative gelatine consumption in male and female Sprague–Dawley rats. The group and sex effects were significantly different for absolute gelatine consumption (group: $F=54.81$; $p<0.0001$; sex: $F=365.25$; $p<0.0001$). There were no significant differences in absolute gelatine consumption between the groups in both males and females for all 10 weeks (Figure 2a,c). Male rats had higher absolute gelatine consumption compared to females from week 4 to week 10 ($p<0.01$; Figure 2a,c).

When gelatine consumption was computed relative to body mass, the group effect was significant ($F=11.78$; $p<0.0001$), however, the sex effect was not ($F=0.48$; $p=0.48$). There were no significant differences in relative gelatine consumption between the groups in males for all 10 weeks (Figure 2b). In females, the relative gelatine consumption was higher in the control and NA groups compared to the SOH + NA group at week 8 ($p=0.02$ and $p=0.03$) and week 9 ($p=0.03$ and $p=0.03$; Figure 2d).

3.4 | Echocardiography

3.4.1 | Cardiac mass, cardiac geometry, and left ventricular systolic function

Table 2 shows the effects of sweetened alcohol and naringin on cardiac mass, cardiac geometry, and left ventricular systolic function in male and female Sprague–Dawley rats. The group and sex effects were significantly different for relative heart masses (group: $F=21.26$, $p<0.0001$; sex: $F=138.72$, $p<0.0001$) and absolute heart masses (group: $F=6.54$, $p<0.0001$; sex: $F=34.03$, $p<0.0001$). The group and sex effects were significantly different for relative LV masses (group: $F=28.42$, $p<0.0001$; sex: $F=195.68$, $p<0.0001$) and absolute LV masses (group: $F=3.62$, $p<0.0001$; sex: $F=18.11$, $p<0.0001$). Male heart and LV masses were heavier ($p<0.0001$) compared to female heart and LV masses. However, female control, NA, and SOH + NA groups had heavier ($p<0.01$) heart mass indexed to body mass compared to male control and SOH groups. Female control, NA, and SOH + NA groups had heavier ($p<0.01$) LV mass indexed to body mass compared to the male control group.

The group and sex effects were significantly different for LV end-diastolic diameter (group: $F=42.75$, $p<0.0001$; sex: $F=297.96$, $p<0.0001$), LV end-diastolic posterior wall thickness (group: $F=6.72$, $p<0.0001$; sex: $F=27.97$, $p<0.0001$), LV end-systolic diameter (group: $F=5.41$, $p<0.0001$; sex: $F=37.47$, $p<0.0001$), LV end-systolic posterior wall thickness (group: $F=8.90$, $p=0.0001$; sex: $F=62.04$, $p<0.0001$), and relative wall thickness (group: $F=5.71$, $p<0.0001$; sex: $F=17.17$, $p<0.0001$) in male and female rats. Female SOH rats had greater LV end-diastolic posterior wall thickness, and relative wall thickness compared to female control rats ($p=0.04$, and $p=0.01$, respectively). Female SOH rats had greater relative wall thickness compared to female NA rats ($p=0.01$). No significant differences in relative wall thickness were observed between the female control, NA, and SOH + NA groups. LV end-diastolic diameter and LV end-systolic posterior wall thickness were greater ($p<0.001$) in all males compared to all female groups. Female control and NA rats had reduced ($p<0.01$) LV end-diastolic posterior wall thickness and LV end-systolic diameter compared to all male groups. Female SOH rats had greater ($p<0.01$) relative wall thickness compared to all male groups. Female SOH + NA rats had greater ($p<0.05$) relative wall thickness compared to male control and NA groups.

The group and sex effects were significantly different for LV stroke volume (group: $F=4.04$, $p=0.0009$; sex: $F=27.56$, $p<0.0001$). There were no significant differences in group or sex effects for ejection fraction (group: $F=0.49$, $p=0.84$; sex: $F=1.19$, $p=0.28$). Although the sex effect was significantly different for fractional shortening ($F=11.84$, $p=0.0010$), no significant differences were observed for the group effect ($F=1.83$, $p=0.11$). There were no significant differences between groups in all markers of systolic function in male and female rats.

3.4.2 | Left ventricular diastolic function

Pulsed Doppler indices

Table 3 shows the effects of sweetened alcohol and naringin on pulsed Doppler indices (E and E/A) in male and female Sprague–Dawley rats. There were no significant differences observed in the group and sex effects for E velocity (group: $F=0.12$, $p=0.99$; sex: $F=0.00$, $p=0.96$) and E/A (group: $F=0.00$, $p=1.00$; sex: $F=0.01$, $p=0.91$) in male and female rats.

Tissue Doppler indices

Figure 3 shows the effects of sweetened alcohol and naringin on tissue Doppler indices in male and female Sprague–Dawley rats. The group and sex effects were significantly different for lateral e' (group: $F=15.41$, $p<0.0001$; sex: $F=52.18$, $p<0.0001$), e'/a' (group: $F=16.94$, $p<0.0001$; sex: $F=61.35$, $p<0.0001$), and E/e'

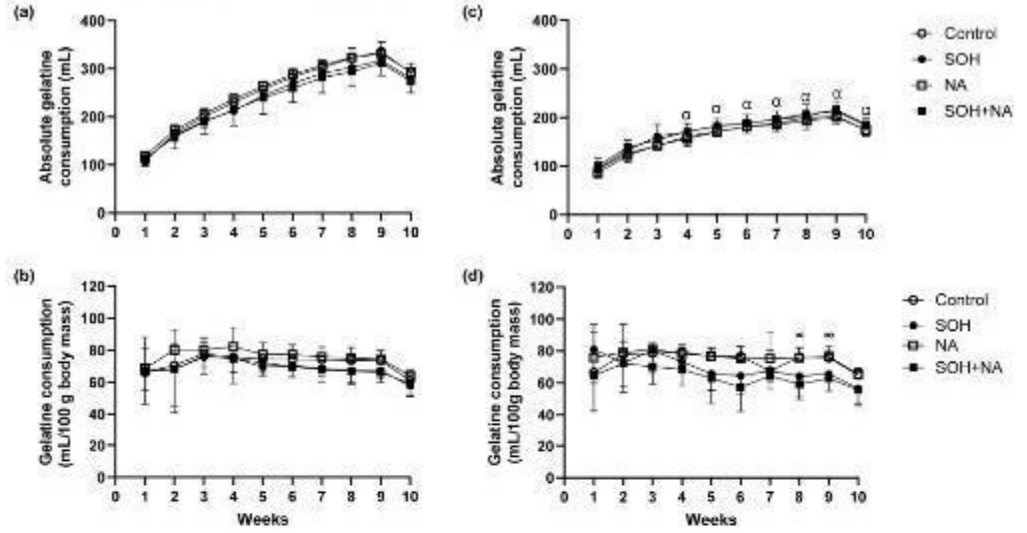

FIGURE 2 The weekly absolute (a and c) and relative (b and d) gelatine consumption in male (left panel) and female (right panel) Sprague–Dawley rats. Data presented as means ± SEM. $^{*}p < 0.05$ control and NA compared to SOH + NA. $^{\#}p < 0.05$ compared to all male groups. NA, naringin; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

(group: $F = 14.25$, $p < 0.0001$; sex: $F = 34.55$, $p < 0.0001$) in male and female rats.

There were no significant differences in all tissue Doppler indices in male rats (Figure 3a–c). Female SOH (mean ± SEM: 3.11 ± 0.29 cm/s) and SOH + NA (mean ± SEM: 3.40 ± 0.28 cm/s) groups had reduced lateral e' compared to control (mean ± SEM: 5.33 ± 0.29 cm/s) and NA (mean ± SEM: 5.30 ± 0.28 cm/s) groups ($p < 0.0001$; Figure 3d). Female SOH (mean ± SEM: 0.64 ± 0.12) and SOH + NA (mean ± SEM: 0.75 ± 0.11) groups had reduced e'/a' compared to control (mean ± SEM: 1.57 ± 0.12) and NA (mean ± SEM: 1.51 ± 0.11) groups (All $p < 0.0001$; Figure 3e). Female SOH (mean ± SEM: 24.21 ± 1.37) and SOH + NA (mean ± SEM: 23.16 ± 1.31) groups had increased E/e' compared to control (mean ± SEM: 13.35 ± 1.37) and NA (mean ± SEM: 13.12 ± 1.31) groups ($p < 0.0001$; Figure 3f). No significant differences were observed between the female SOH and SOH + NA groups.

All male groups had higher e', e'/a' and reduced E/e' compared to female SOH and SOH + NA groups (All $p < 0.0001$).

3.5 | Cardiomyocyte diameters

Figure 4 shows the effects of sweetened alcohol and naringin on LV cardiomyocyte diameters in longitudinal sections in male and female Spraque-Dawley rats. The group and sex effects were significantly different for the LV cardiomyocyte diameters (group: $F = 14.13$, $p < 0.0001$; sex: $F = 75.39$, $p < 0.0001$) in male and female rats. There were no statistical differences in LV cardiomyocyte diameters between the groups in male rats (Figure 4a,b). In females, LV cardiomyocyte diameters were significantly greater in the SOH (mean ± SEM: 17.79 ± 0.38 μm) compared to the control (mean ± SEM: 15.71 ± 0.38 μm; $p = 0.005$) and NA groups (mean ± SEM: 15.81 ± 0.38 μm; $p = 0.008$; Figure 4c,d). All male groups had greater LV cardiomyocyte diameters compared to the female control, NA, and SOH + NA groups ($p < 0.05$).

3.6 | Collagen area fraction

Figure 5 shows the effects of sweetened alcohol and naringin on LV collagen area fraction in male and female Sprague–Dawley rats. There were no visual differences in collagen accumulation between the groups of male rats when observed under bright filled (Figure 5a) or polarized light (Figure 5c). There was greater collagen accumulation in female SOH and SOH + NA rats compared to control and NA rats as evidenced by the increased red staining of cardiac tissue section visualized under bright-filled microscopy (Figure 5b) and large collagen fibers in orange or yellow visualized under polarized light (Figure 5d).

TABLE 2 Effects of sweetened alcohol and naringin on cardiac masses and geometry, and left ventricular systolic function in male and female Sprague–Dawley rats.

	Males				Females			
	Control	SOH	NA	SOH + NA	Control	SOH	NA	SOH + NA
Cardiac masses and geometry								
Heart mass (g)	1.63 ± 0.06	1.55 ± 0.06	1.80 ± 0.06	1.61 ± 0.06	1.14 ± 0.02[a]	1.19 ± 0.10[a]	1.11 ± 0.04[a]	1.16 ± 0.05[a]
Heart mass/body mass x 10^3	3.52 ± 0.12[a]	3.57 ± 0.13	3.52 ± 0.20[a]	3.90 ± 0.12	4.41 ± 0.09	4.03 ± 0.09	4.32 ± 0.19	4.33 ± 0.12
LV mass (g)	0.68 ± 0.03	0.72 ± 0.03	0.72 ± 0.02	0.73 ± 0.02	0.47 ± 0.02[a]	0.48 ± 0.02[a]	0.49 ± 0.03[a]	0.50 ± 0.02[a]
LV mass/body mass $\times 10^3$	1.48 ± 0.07[a,c]	1.67 ± 0.07	1.59 ± 0.05	1.73 ± 0.04	1.83 ± 0.06	1.70 ± 0.06	1.89 ± 0.11	1.82 ± 0.08
LV end-diastolic diameter (mm)	7.90 ± 0.16	7.33 ± 0.16	7.46 ± 0.17	7.41 ± 0.16	5.46 ± 0.17[a]	5.29 ± 0.17[a]	5.43 ± 0.16[a]	5.32 ± 0.16[a]
LV end-diastolic posterior wall thickness (mm)	1.70 ± 0.07	1.78 ± 0.07	1.66 ± 0.07	1.72 ± 0.08	1.27 ± 0.08[a]	1.62 ± 0.08[a,b]	1.31 ± 0.07[a]	1.56 ± 0.07
LV end-systolic diameter (mm)	3.86 ± 0.16	3.87 ± 0.16	3.88 ± 0.16	3.89 ± 0.17	3.11 ± 0.17[a]	3.19 ± 0.17	3.09 ± 0.16[a]	3.22 ± 0.16
LV end systolic posterior wall thickness (mm)	3.12 ± 0.11	3.11 ± 0.11	3.06 ± 0.11	3.12 ± 0.11	2.51 ± 0.11[a]	2.48 ± 0.11[a]	2.51 ± 0.11[a]	2.49 ± 0.11[a]
Relative wall thickness	0.46 ± 0.02[b]	0.49 ± 0.02	0.45 ± 0.02[b]	0.47 ± 0.03	0.47 ± 0.03	0.62 ± 0.03[a,c]	0.49 ± 0.02	0.57 ± 0.02
Systolic function								
LV stroke volume (mL)	0.53 ± 0.03	0.50 ± 0.03	0.53 ± 0.03	0.51 ± 0.03	0.41 ± 0.03	0.40 ± 0.03	0.40 ± 0.03	0.40 ± 0.03
Ejection fraction (%)	80.31 ± 2.19	78.27 ± 2.19	78.74 ± 2.19	78.21 ± 2.31	81.99 ± 2.31	79.74 ± 2.31	82.04 ± 2.19	78.76 ± 2.19
Fractional shortening (%)	48.31 ± 2.51	46.94 ± 2.51	47.93 ± 2.51	47.29 ± 2.65	42.87 ± 2.65	39.74 ± 2.65	41.88 ± 2.51	41.02 ± 2.51

Note: Data presented as mean ± STM. [a]$p < 0.05$ compared to control. [b]$p < 0.05$ compared to NA. [c]$p < 0.05$ compared to all male groups. [d]$p < 0.05$ compared to female SOH + NA group. [e]$p < 0.05$ compared to female control, NA and SOH + NA.

Abbreviations: LV, left ventricle; NA, naringin; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

TABLE 3 Effects of sweetened alcohol and naringin on left ventricular diastolic function (pulsed Doppler indices) in male and female Sprague–Dawley rats.

	Males				Females			
	Control	SOH	NA	SOH + NA	Control	SOH	NA	SOH + NA
E (cm/s)	70.75 ± 3.13	73.20 ± 3.13	70.70 ± 3.13	70.89 ± 3.30	70.00 ± 3.30	72.06 ± 3.30	70.60 ± 3.13	72.50 ± 3.13
E/A	1.67 ± 0.13	1.65 ± 0.13	1.66 ± 0.13	1.65 ± 0.14	1.68 ± 0.14	1.66 ± 0.14	1.66 ± 0.13	1.67 ± 0.13

Note: Data presented as means ± SEM.

Abbreviations: A, late diastolic inflow velocities; E, early; E/A, ratio of early and late diastolic inflow velocities; LV, left ventricle; NA, naringin; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

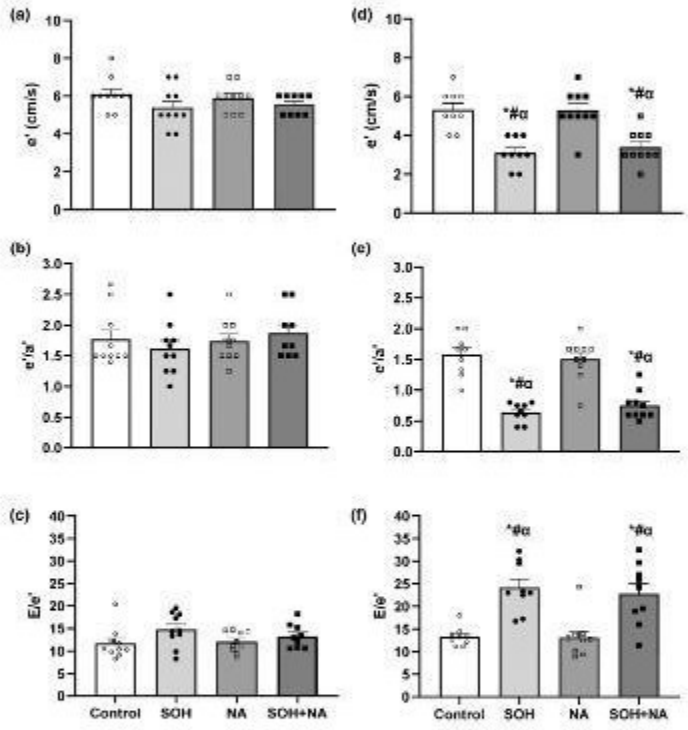

FIGURE 3 Effects of sweetened alcohol and naringin on lateral e' (a and d), e'/a' (b and e), and E/e' (c and f) in male (left panel) and female (right panel) Sprague–Dawley rats. Data presented as mean ± SEM. *$p < 0.0001$ compared to control. #$p < 0.0001$ compared to NA. α$p < 0.0001$ compared to all male groups. LV, left ventricular; NA, naringin; SOH, sweetened alcohol; SOH + NA, sweetened alcohol and naringin. e', peak velocity during early diastole; e'/a'; ratio of early to late peak velocities during diastole; E/e', ratio of early diastolic filling to peak velocity during early diastole.

The group and sex effects were significantly different for the collagen area fraction (group: $F = 40.53$, $p < 0.0001$; sex: $F = 87.89$, $p < 0.0001$) in male and female rats. There were no significant differences in the collagen area fraction between the groups in male rats (Figure 5e). In females, the collagen area fraction was significantly greater in the SOH group (mean ± SEM: 9.12 ± 0.52%) compared to the control (mean ± SEM: 1.58 ± 0.52%; $p < 0.0001$), and NA (mean ± SEM: 2.15 ± 0.51%; $p < 0.0001$) groups (Figure 5f). In females, the collagen area fraction was significantly greater in the SOH + NA group (mean ± SEM: 8.71 ± 0.47%) compared to the control (mean ± SEM: 1.58 ± 0.52%; $p < 0.0001$) and NA (mean ± SEM: 2.15 ± 0.51%; $p < 0.0001$) groups

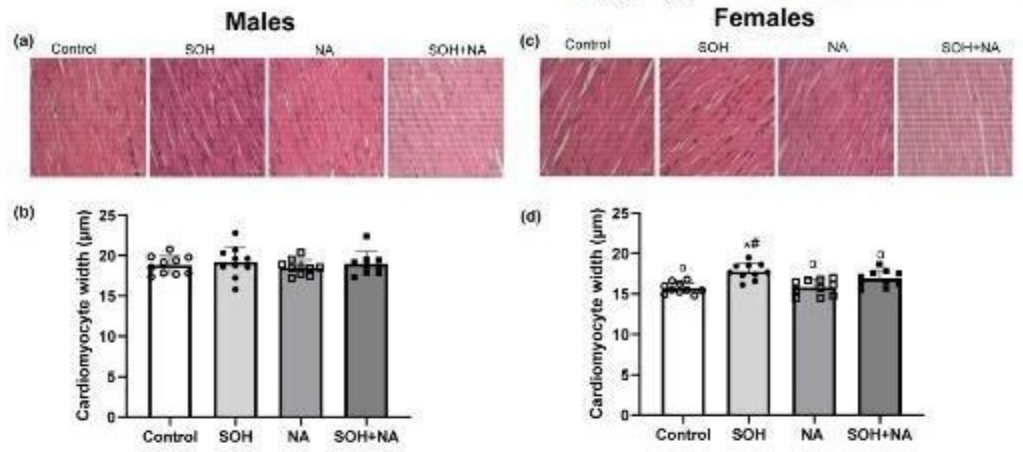

FIGURE 4 Effects of sweetened alcohol and naringin on LV cardiomyocyte diameters in longitudinal sections of male (left panel) and female (right panel) Sprague–Dawley rats. Representative H&E-stained micrographs imaged at ×40 objective (×400 magnification) viewed in bright-field (a and c). LV cardiomyocyte diameters (b and d) measured from the H&E stained sections. Data presented as means ± SEM. *$p < 0.05$ compared to control. $^{\#}p < 0.05$ compared to NA. $^{\alpha}p < 0.0001$ compared to all male groups. LV, left ventricle; NA, naringin; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

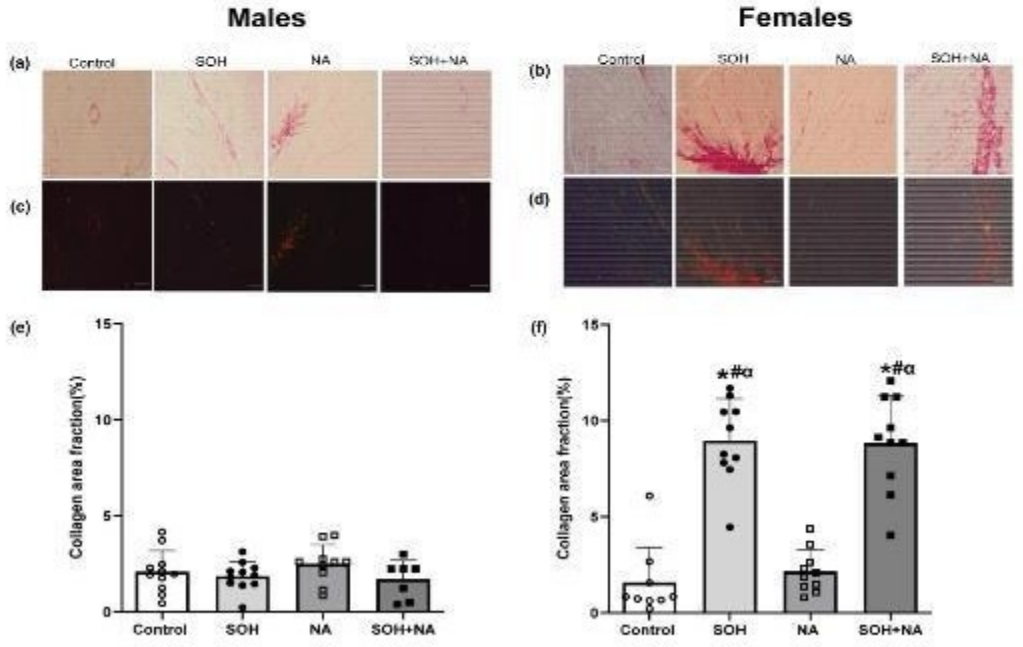

FIGURE 5 Effects of sweetened alcohol and naringin on collagen area fraction in LV tissue of male and female Sprague–Dawley rats. Representative picrosirius red stained micrographs imaged at ×10 objective (×100 magnification) viewed in bright-field (a and b) and under polarized light (c and d). Collagen area fraction (e and f) calculated from picrosirius red-stained sections. Data presented as means ± SEM. *$p < 0.05$ compared to control. $^{\#}p < 0.05$ compared to NA. $^{\alpha}p < 0.05$ compared to all male groups. LV, left ventricle; NA, naringin; SOH + NA, sweetened alcohol and naringin; SOH, sweetened alcohol.

(Figure 5f'). No significant differences in collagen area fraction were observed between SOH and SOH + NA female groups ($p = 0.99$; Figure 5f).

All male groups had reduced collagen area fraction compared to female SOH and SOH + NA groups (All $p < 0.0001$).

3.7 | Associations between the collagen area fraction and tissue Doppler indices

Figure 6 shows the associations between the collagen area fraction and tissue Doppler indices. Reduced e' was associated with increased collagen area fraction ($r = -0.35$; $p = 0.0018$). Reduced e'/a' was associated with collagen area fraction ($r = -0.31$; $p = 0.0069$). Increased E/e' was associated with collagen area fraction ($r = 0.33$; $p = 0.0039$; Figure 6a). After adjusting for blood pressure, the results remained materially unaltered (e': partial $r = -0.34$ and $p = 0.0033$; e'/a': partial $r = -0.30$ and $p = 0.0147$; E/e': partial $r = 0.31$ and $p = 0.0095$; Figure 6b).

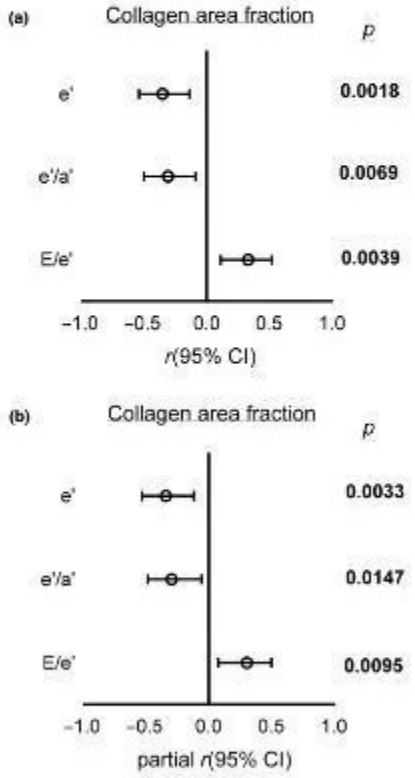

FIGURE 6 Associations between the collagen area fraction and measures of left ventricular diastolic function. (a) Before adjusting for BP, (b) After adjusting for BP. e', peak velocity during early diastole; e'/a', ratio of early to late peak velocities during diastole; E/e', ratio of early diastolic filling to peak velocity during early diastole. Open circles represent the correlation coefficient (r or partial r), and horizontal lines represent the 95% confidence intervals (CI).

4 | DISCUSSION

The current study investigated the effects of sweetened alcohol and naringin on cardiac function in male and female adolescent Sprague–Dawley rats. The main findings of this study were that sweetened alcohol and naringin did not affect gelatine consumption in male rats. However, in female rats, the combination of sweetened alcohol and naringin led to decreased gelatine consumption. In males, sweetened alcohol or naringin did not exert an impact on cardiac geometry, systolic function, or diastolic function. However, in females, the consumption of sweetened alcohol led to concentric remodeling without inducing alterations in LV mass and blood pressure. Although sweetened alcohol did not affect systolic function, it impaired LV relaxation and led to elevated LV filling pressures in females. Increased collagen area fraction was associated with impaired LV relaxation and elevated filling pressures. Naringin may have the potential to improve the sweetened alcohol-induced concentric remodeling, but not diastolic dysfunction in females. These findings highlight potential sex-specific differences in cardiac structure and function in response to exposure to sweetened alcohol and naringin.

4.1 | Effects of sweetened alcohol and naringin on gelatine consumption

Relative gelatine consumption, which accounts for body weight, was not significantly different among the male groups. This indicates that when adjusted for body mass, the male rats consumed gelatine in similar proportions regardless of their experimental condition. This uniformity suggests that the interventions did not markedly affect the dietary behavior of male rats in terms of gelatine consumption. In contrast, significant differences were noted in the relative gelatine consumption among female rats. Specifically, females in the SOH + NA group had reduced relative gelatine consumption compared to both the control and NA groups in week 8 and 9 of the study, however, was similar in the 10th week. It is unclear why this transient reduction in gelatine consumption in the SOH + NA group occurred. It does suggest

that the combination of SOH and NA might influence the feeding behavior in female rats. Indeed, differences in preference for alcohol and fructose have previously been reported in rats (Li et al., 2019; Nieto & Kosten, 2017).

4.2 | Effects of sweetened alcohol on cardiac geometry

In the current study, no differences were observed in markers of cardiac geometry in adolescent male rats. However, in adolescent females, the consumption of sweetened alcohol increased LV end-diastolic posterior wall thickness and relative wall thickness compared to controls without altering heart and LV masses. In addition, the consumption of sweetened alcohol marginally increased the LV cardiomyocyte diameters. These results suggest that the consumption of sweetened alcohol may have caused concentric remodeling in females. Indeed, in concentric remodeling, LV wall thickness and relative wall thickness increase before any detectable changes in LV mass. The individual effects of a high-fructose diet and alcohol on cardiac remodeling are controversial. While some studies have reported eccentric cardiac remodeling characterized by reduced LV wall thickness and relative wall thickness in rats subjected to fructose (Bouchard-Thomassin et al., 2011; Komnenov et al., 2020) or alcohol (Ai et al., 2020; Kim et al., 2003; Mouton et al., 2020), other studies have reported concentric cardiac remodeling and hypertrophy in rats exposed to fructose (Wang et al., 2023; Xing et al., 2010) or alcohol (Fernández-Solà & Porta, 2016). Interestingly, Wu et al. (2018) reported that the coadministration of fructose and alcohol (6% fructose +4% ethanol) led to improvements in myocardial hypertrophy compared to rats only consuming a high fructose diet (6% fructose) (Wu et al., 2018). The discrepancies in these findings may be due to specific variations in dietary conditions, age, as well as differences in rat strain and sex. Nevertheless, in the current study, we found that long-term consumption of sweetened alcohol may result in more pronounced LV mass changes and concentric hypertrophy in females.

Although hypertension is a significant contributor to the cardiac remodeling process (Tomek & Bub, 2017), in the present study, blood pressures were similar between the control and SOH groups. This suggests that concentric remodeling in females occurred independent of changes in blood pressure. Indeed, cardiac remodeling can be induced not only by hypertension but also by inflammatory and fibrotic responses (Wang et al., 2023). In the current study, the consumption of sweetened alcohol increased LV total collagen content compared to the control group. Indeed, high fructose (Wang et al., 2023; Xing et al., 2010) or alcohol consumption (Lluís et al., 2011; Mouton et al., 2020) have been associated with cardiac fibrosis. In the heart, both fructose and alcohol may induce oxidative stress and inflammation, which then activate pathways that promote increased collagen formation and fibrosis (Kang et al., 2015; Zhang et al., 2016). Therefore, our findings suggest that the consumption of sweetened alcohol resulted in increased myocardial fibrosis which could have led to concentric remodeling in females.

4.3 | Effects of sweetened alcohol on diastolic function

In the current study, sweetened alcohol did not affect pulsed Doppler indices in male and female Sprague-Dawley rats. In agreement with our study, Wu et al. (2018) reported no changes in the E/A ratio in 8-week-old rats exposed to sweetened alcohol (6% fructose +4% ethanol) for 8 weeks (Wu et al., 2018). Despite no changes in pulsed Doppler indices, the consumption of sweetened alcohol in the current study led to impaired tissue Doppler indices including reduced lateral e' and e'/a' and increased E/e' in female rats. In addition, increased collagen area fraction was associated with impaired LV relaxation and elevated filling pressures.

Although Wu et al. (2018) did not report on tissue Doppler indices, previous studies have reported impaired LV relaxation (reduced lateral e') and increased filling pressures (E/e') in rats fed a high fructose diet (Xing et al., 2010) and alcohol (Catena et al., 2016). In this regard, the consumption of sweetened alcohol may have impaired passive LV relaxation by promoting excessive collagen production and deposition that in turn, increased myocardial fibrosis. The increased myocardial fibrosis and impaired LV relaxation and stiffness caused by the sweetened alcohol may therefore lead to increased LV filling pressures (E/e'). In the current study, sweetened alcohol increased LV pressures despite the normal appearance of the E/A ratio. Indeed, increases in LV filling pressures drive a compensatory increase in the early diastolic filling (E), resulting in a pseudonormal filling pattern. Therefore, in the present study, the consumption of sweetened alcohol most likely caused moderate-to-severe diastolic dysfunction.

The mechanisms by which fructose and alcohol impair LV relaxation and increase filling pressures are multifactorial. In addition to promoting fibrosis-mediated myocardial stiffness, fructose and alcohol may contribute to diastolic dysfunction by modifying calcium homeostasis, impacting contractile proteins, inducing oxidative

stress, increasing lipogenesis, and promoting lipid accumulation in the myocardium (Kang et al., 2015; Laonigro et al., 2009; Li et al., 2022; Rasoul et al., 2023; Tsermpini et al., 2022; Wang et al., 2015, 2023; Zhang et al., 2016). Future studies should investigate these molecular mechanisms in more detail to gain a deeper understanding of their specific contributions.

4.4 | Effects of sweetened alcohol on systolic function

In this study, the consumption of sweetened alcohol and naringin did not affect systolic function in both male and female rats. While some studies have reported that the individual consumption of fructose (Wang et al., 2023; Zhang et al., 2016) and alcohol (Ai et al., 2020; Kim et al., 2003; Mouton et al., 2020; van Oort et al., 2020) can adversely affect systolic function, characterized by impaired stroke volume, ejection fraction, and fractional shortening, others have not (Wang et al., 2023; Zhang et al., 2016). The majority of studies that have reported reduced ejection fraction and fractional shortening (Ai et al., 2020; Li et al., 2016; Wang et al., 2023; Zhang et al., 2016) have administered fructose and alcohol at high doses, and for a longer duration of intervention, which could partly explain the discrepancies in the results.

Nonetheless, similar to our results, Wu et al. (2018) reported no changes in systolic function in rats receiving sweetened alcohol. The assessment of systolic function in clinical practice often relies on LV ejection fraction, a measure influenced by chamber size and pressure. As a result, it serves as a more reliable indicator for evaluating ventricular-arterial coupling than contractility (Borlaug et al., 2009). In our study, the use of more sensitive echocardiographic parameters, specifically Speckle Tracking Imaging (Dandel et al., 2009), may have facilitated the early detection of LV systolic dysfunction. In this context, research has indicated that myocardial deformation properties, specifically LV circumferential and longitudinal strain and strain rate may be compromised before any noticeable changes in systolic chamber function (Kucuk et al., 2017). Future studies should therefore use more sensitive measures of systolic function to explore the effect of sweetened alcohol on systolic performance.

4.5 | Sex-specific effects of sweetened alcohol on cardiac remodeling and diastolic function

Estrogen possesses anti-inflammatory and vasodilatory properties, producing a cardioprotective environment for women (Iorga et al., 2017; Xiang et al., 2021). Nevertheless, our study showed that sweetened alcohol caused concentric remodeling and diastolic dysfunction in female rats, and not in their male counterparts. One plausible explanation for this discrepancy may be due to the interplay between sweetened alcohol and other physiological factors, specifically visceral adiposity. In the current study, female SOH rats had increased visceral adiposity indexed to body mass compared to male rats. Indeed, studies have reported that fructose consumption has a more pronounced effect on visceral adiposity in female rats compared to males (Kovačević et al., 2021). In addition, the accumulation of excess visceral adipose tissue plays an important role in the development of diastolic dysfunction and heart failure with a preserved ejection fraction in women more than in men (Sorimachi et al., 2021).

Hypertrophic adipocytes, prevalent in visceral adiposity may secrete inflammatory markers such as tumor necrosis factor-alpha, interleukin 1β, interleukin 6, adipokines such as adiponectin, leptin, resistin, chemotactic protein 1 (MCP-1), and transforming growth factor (TGF)-β, (Farkhondeh et al., 2020) thereby promoting a state of chronic low-grade inflammation (Kolb, 2022). The increased circulating levels of these inflammatory markers may cause damage to the endothelium leading to the upregulation of soluble intercellular adhesion molecules that increase the production of ROS (Paulus & Tschöpe, 2013). ROS decreases the bioavailability of nitric oxide and increases the production of peroxynitrites (Schulz et al., 2008). This in turn reduces the production of cyclic guanosine monophosphate and lowers protein kinase G (PKG) activity within the myocardium (van Heerebeek et al., 2012). These altered signaling pathways and PKG activity result in increased cardiac remodeling, diastolic stiffness, and dysfunction through downstream hypophosphorylation of the protein, titin (Paulus & Tschöpe, 2013). This ultimately leads to impaired diastolic function and concentric cardiac remodeling. Therefore, the sweetened alcohol-induced increased visceral adiposity may have triggered a cascade of inflammatory events leading to the remodeling process in females, thus, increasing their susceptibility to diastolic dysfunction.

These results shed light on the complexity of gender-specific responses to sweetened alcohol-induced cardiac effects and highlight the importance of gaining a better understanding of the underlying physiological mechanisms contributing to diastolic function impairment and cardiac remodeling in female rats. Further studies are crucial to elucidate the interactions between visceral adiposity, inflammation, and other contributing factors that collectively influence the observed gender-specific outcomes in response to sweetened alcohol administration.

4.6 | Effects of naringin on sweetened alcohol-induced concentric remodeling and diastolic dysfunction

In the current study, naringin may have potentially improved the sweetened alcohol-induced concentric remodeling as the LV posterior wall thickness, relative wall thickness, and LV cardiomyocyte diameters in the SOH+NA group was similar to that of the controls. Previous studies have shown the potential therapeutic and cardioprotective effects of naringin in various experimental models of diet-induced cardiac injury, diabetic cardiomyopathy, and ischemic heart diseases (Malakul et al., 2018; Park et al., 2018; Viswanatha et al., 2022).

Park et al. (2018) reported that naringin prevented the generation of ROS and mitochondrial dysfunction in cardiomyocytes exposed to fructose, which in turn decreased the hypertrophy of the cardiomyocytes (Park et al., 2018). Moreover, naringin prevented fructose-induced cardiomyocyte apoptosis by obstructing ROS-dependent ATM-mediated p53 signaling (Park et al., 2018). To the best of our knowledge, the cardioprotective effects of naringin have not been explored in alcoholic models.

Although naringin may have marginally improved the sweetened alcohol-induced concentric remodeling, it did not improve the severity of fibrosis since the total collagen content was similar in SOH+NA and SOH groups but were both significantly higher compared to controls. Therefore, it is plausible that naringin did not directly impact collagen formation, but rather it may have improved indices of concentric remodeling through alternative mechanisms, such as the reduction in ROS formation as reported in other studies (Akin et al., 2022; Bruno et al., 2022; Laonigro et al., 2009). Future studies should consider these mechanisms to further explore the potential of naringin in the improvement of sweetened alcohol-induced concentric remodeling at a molecular level.

Although naringin may have marginally improved concentric remodeling, it did not ameliorate the sweetened alcohol-induced diastolic dysfunction. Concentric remodeling has the potential to contribute to diastolic dysfunction, as the increased stiffness of the heart muscle may affect its ability to relax during diastole (Røe et al., 2017). Given the observed improvement in sweetened alcohol-induced concentric remodeling with naringin, a longer duration of treatment and higher doses of naringin may have eventually led to improvements in diastolic function. Therefore, these factors should be considered in future studies. Additionally, understanding the broader metabolic responses that lead to reduced gelatine consumption in females could provide insights into the interconnected nature of diet and cardiac health in the presence of such substances.

The present study has further limitations. While previous studies have extensively examined the individual effects of fructose (Bouchard-Thomassin et al., 2011; Kang et al., 2015; Komnenov et al., 2020; Wang et al., 2023; Xing et al., 2010; Zhang et al., 2016) and alcohol (Ai et al., 2020; Fernández-Solà & Porta, 2016; Kim et al., 2003; Mouton et al., 2020; Nieto & Kosten, 2017) on cardiac function, these studies did not employ gelatine as a vehicle. Future investigations would benefit from the incorporation of 20% fructose and 10% ethanol solely within gelatine to provide a clearer comparison between individual and combined effects. In addition, the use of a single dose or concentration for each compound of interest (fructose at 20%, alcohol at 10%, and naringin at 50 mg/kg) may have contributed to the absence of some of the observable effects. Future investigations need to explore a wider range of doses to elucidate potential dose–response relationships more comprehensively. As Gomori's trichrome and wheat germ agglutinin (WGA) stains offer better specificity and quantitative accuracy for measuring cardiomyocyte size compared to the more general H&E staining, further investigation in this regard may provide additional insights in the assessment of cardiac remodeling. Finally, the lack of measurement of blood alcohol concentration following consumption of ethanol-containing gelatine is an aspect that warrants further investigation in future studies since insights into how gelatine matrices influence alcohol release and absorption can inform alcohol metabolism considerations and health implications.

5 | CONCLUSION

In males, neither sweetened alcohol nor naringin had any impact on cardiac geometry or diastolic function. Conversely, in females, the consumption of sweetened alcohol resulted in increased visceral obesity, and concentric remodeling without inducing alterations in LV mass and blood pressure. Sweetened alcohol-impaired LV relaxation and elevated LV filling pressures in females. Notably, naringin improved the sweetened alcohol-induced concentric remodeling but did not mitigate the diastolic dysfunction and visceral obesity induced by sweetened alcohol in females. These findings contribute to a better understanding of the effects of sweetened alcohol and naringin on cardiac health and underscore the need for further research to unravel the underlying mechanisms that account for gender-specific differences.

AUTHOR CONTRIBUTIONS

JM collected and analyzed the data and drafted the manuscript. KHE, KGI designed the research, collected the data, and reviewed the manuscript. LM designed the research, collected, and analyzed the data, and reviewed the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

The study adhered to international ethical guidelines and standards governing the care and utilization of laboratory animals, receiving approval from the Animal Research Ethics Committee of the University of Witwatersrand (AREC clearance certificate number 2022/10/02/C). The research took place at the Wits Research Animal Facility situated at the University of the Witwatersrand in Johannesburg, South Africa.

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APPENDIX 5A: ELISA PROTOCOL FOR SERUM INSULIN DETERMINATION

ELISA PROTOCOL FOR INSLIN DETERMINATION

Reagent preparation

All reagents were brought to room temperature before use

Standard: Was rreconstitute with 0.5mL of Standard Diluent, kept for 10 minutes at room temperature, Shaked gently. The concentration of the standard in the stock solution was 1,000 µg/mL. Five tubes were prepared containing 0.6mL Standard Diluent. A quadruple dilution series was prepared as shown in figure below.

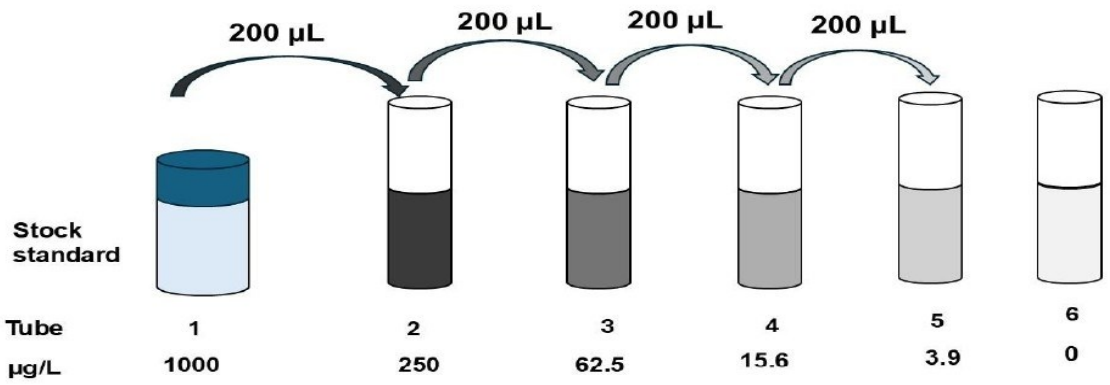

Figure: Tubes preparation and dilution

Each tube was thoroughly vortexed before the next transfer. Five points of diluted standard (1,000µg/mL, 250µg/mL, 62.5µg/mL, 15.6µg/mL, 3.9µg/mL, and the last EP tubes with Standard Diluent is the blank as 0µg/m) were set up.

Detection Reagent A and Detection Reagent B - Detection A and Detection B reagents were briefly centrifuged before use. Diluted to the working concentration 100-fold with **Assay Diluent A** and **B** respectively.

Wash Solution – Deionized water at 580 mL was used Dilute 20 mL of Wash Solution concentrate (30×) to prepare 600 mL of Wash Solution (1×).

TMB (3,3',5,5'- Tetramethylbenzidine) substrate – the needed dosage of the solution was aspirated using sterilized tips.

ASSAY PROCEDURE

1. Wells for diluted standard, blank and sample were determined. Five wells for standard points, 1 well for blank were prepared.
2. Fifty micro litres (50µL) each of dilutions of standard, blank and samples were added into the appropriate wells respectively.
3. Then, 50 µL of Detection Reagent A was added to each well immediately. The plate was shacked gently using a microplate shaker. The plate was covered with a Plate sealer. Incubated for 1 hour at 37ºC.
4. The solution was aspirated and washed with 350µL of 1X Wash Solution to each well using a multi-channel pipette and was allowed to sit for 1-2 minutes. The remaining liquid from all wells was completely removed by snapping the plate onto absorbent paper. This was repeated 3 times. After the last wash, any remaining wash Buffer was removed by aspirating or decanting. The plate was Inverted and blotted against absorbent paper.

3. one hundred micro litres (100µL) of Detection Reagent B working solution was added to each well. Incubated for 30 minutes at 37ºC after it was covered with plate sealer provided by the manufacturer.

4. The aspiration/wash process was repeated 5 times as conducted in step 4 above.

5. Ninety micro litres (90µL) of Substrate Solution was added to each well. Covered with a new Plate sealer and incubated for 10 - 20 minutes at 37ºC. it was protected from light by covering it with foil paper. Protect from the light. A substrate solution was then added which turned the liquid blue.

6. Fifty micro litres (50µL) of Stop Solution were added to each well, and the liquid turned yellow. The liquid was mixed by tapping the side of the plate.

7. Any drop of water and fingerprint was removed from the bottom of the plate and ensured that there are no bubbles on the surface of the liquid.

8. reading was done on the microplate reader at 450 nm immediate

APPENDIX 5B: PROTOCOL FOR TRIGLYCERIDE DETERMINATION USING ELISA

ELISA PROTOCOL FOR TRIGLYCERIDE DETERMINATION

Reagent preparation

1. All reagents were brought to room temperature before uses
2. The stored serum sample was centrifuged at 1000 x g for 5 minutes
3. **Wash buffer:** to prepare 750 ml of a wash buffer, 30 ml of concentrated wash buffer was diluted with 720ml of deionized water. To make sure there was no crystal left, the buffer was gently warm in a 40°C water bath and gently mixed until all the crystals dissolved.
4. **Standard working solution:** the standard was centrifuged at 10, 000 x g for 1 minute, 1 ml of reference standard and sample diluent was added and allowed to stand for 10 minutes, it was gently inverted several times. When it fully dissolved, it was thoroughly mixed with pippete. This produced a working solution of 400 pg/ml (Standard working solution).

Dilution method: seven Eppendorf tubes were used. Five hundred micro litre (500 µL) of the 400 pg/ml standard working solution was pipetted to the first tube labelled tube1 and mixed up to produce 200 pg/ml working solution. Then, 500 µL of the sample diluent were added into the remaining tubes labelled 2-7. A serial dilution was made by transferring 500 µL from tube 1 to tube 2, and from tube 2 to tube 3 up to tube 6. Each tube was thoroughly mixed before transferring to the next tube. Tube 1 served as the high standard, sample diluent in the last tube (tube 7) served as a blank, as shown in the figure below.

The dilution gradient ws kept at 400, 200, 100, 50, 25, 12.5, 6.25, 0 from the standard working solution to the blank respectively.

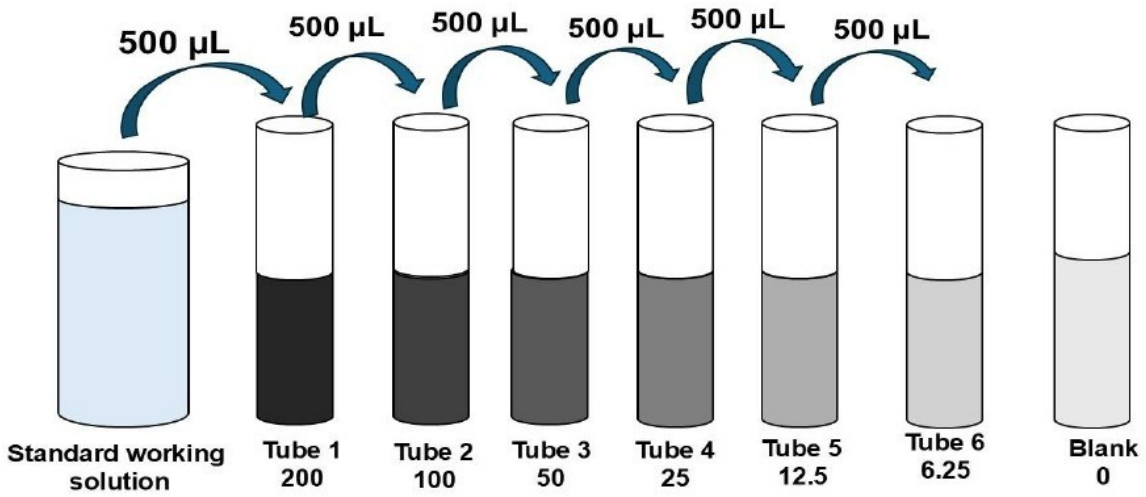

Figure: **Eppendorf Tubes preparation/diluition**

5. **Biotinylated detection antibody working solution**: the amount of the solution needed for the experiment was calculated at about 100µL per well before preparation. However, slightly more than the amount required was prepared. The concentrated biotinylated detection antibody was centrifuged at 800 x g for 1 minute, it was then diluted at 100 x concentrated biotinylated detection antibody to 1 x working solution with biotinylated detection antibody diluent
6. **HRP (Horseradish Peroxidase) Conjugate working solution**: the amount of the solution needed for the experiment was calculated at about 100µL per well before preparation. However, a slightly more than amount required was prepared. The concentrated HRP conjugate was centrifuged at 800 x g for 1 minute, it was then diluted at 100 x concentrated HRP conjugate to 1 x working solution with HRP conjugate.

Assay preparation

1. All samples and standard to be assayed were done in duplicate
2. The wells for diluted standard, blank and sample were determined

3. One hundred micro litre (100 µL) each of the standard, blank and sample were added into the appropriate wells. The plate was covered with a sealer provided by the manufacturer. It was incubated for 90 minutes at 37ºC. (to avoid foaming as much as possible, the solution was carefully added to the bottom of the micro-ELISA plate well and touching of the inside wall was avoided).
4. The liquid from each well was decanted. Without washing, 100 µL of the biotinylated detection antibody working solution was added to each well. The plate was sealed with a new sealer and incubated for 1 hour at 37ºC.
5. The solution was again decanted from each well. 350 µL of wash buffer was added to each well. Soaked for 1 minute and aspirated the solution from each well. It was then patted to dry against a clean absorbent paper. This wash step was repeated 3 times.
6. One hundred micro litre (100 µL) of HRP conjugate working solution was added to each well. The plate was covered with a new sealer and incubated for 30 minutes at 37ºC.
7. The solution was decanted from each well, and the wash process was repeated 5 times as conducted as done in step 5 above.
8. Ninety micro litres (90 µL) of substrate reagent was added to each well. The plate was covered with a new sealer and incubated for 15 minutes at 37ºC. A foil paper was used to cover the plate to protect it from light. The plate was pre-heated for 15 minutes before optical density determination.
9. Fifty micro litres (50 µL) of stop solution was added to each well
10. The optical density (OD) of each well was determined once with microplate reader set at 450 nm.

APPENDIX 6A: PROTOCOL FOR NGAL DETERMINATION USING ELISA

Reagent preparation

1. All reagents were brought to room temperature before use
2. The stored serum sample was centrifuged at 1000 x g for 5 minutes
3. **Wash buffer:** Seven hundred and fifty milli-litres (750 mL) of wash buffer was prepared by diluting 30 ml of concentrated wash buffer with 720ml of deionized water. To make sure there was no crystal left, the buffer was gently warm in a 40ºC water bath and gently mixed until all the crystals dissolved.
4. **Standard working solution:** the standard was centrifuged at 10, 000 x g for 1 minute, 1 ml of reference standard and sample diluent was added and allowed to stand for 10 minutes, it was gently inverted several times. When it fully dissolved, it was thoroughly mixed with pippete. This produced a working solution of 4000 pg/ml (Standard working solution).

Dilution method: seven Eppendorf tubes were used. Five hundred micro litre (500 µL) of the 4000 pg/ml standard working solution was pipetted to the first tube labelled tube1 and mixed up to produce 2000 pg/ml working solution. Then, 500 µL of the sample diluent were added into the remaining tubes labelled 2-7. A serial dilution was made by transferring 500 µL from tube 1 to tube 2, and from tube 2 to tube 3 up to tube 6. Each tube was thoroughly mixed before transferring to the next tube. Tube 1 served as the high standard, sample diluent in the last tube (tube 7) served as a blank, as shown in figure below.

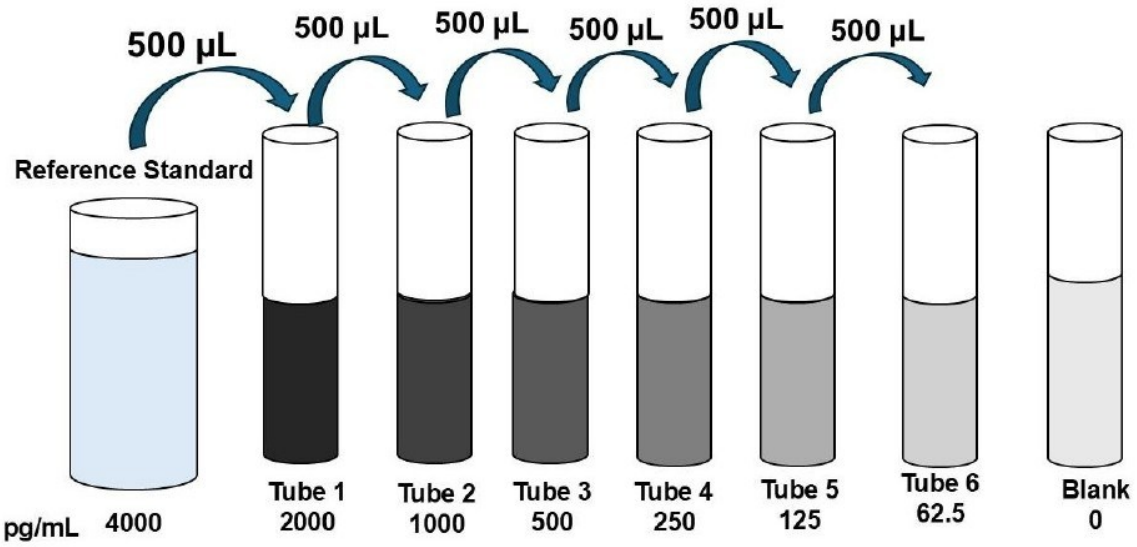

Figure: Tubes preparation and dilution

The dilution gradient was kept at 4000, 2000, 1000, 500, 250, 125, 62.5, 0 pg/mL from the standard working solution to the blank respectively.

5. **Biotinylated detection antibody working solution**: the amount of the solution needed for the experiment was calculated at about 100µL per well before preparation. However, slightly more than the amount required was prepared. The concentrated biotinylated detection antibody was centrifuged at 800 x g for 1 minute, it was then diluted at 100 x concentrated biotinylated detection antibody to 1 x working solution with biotinylated detection antibody diluent
6. **HRP (Horseradish Peroxidase) Conjugate working solution**: the amount of the solution needed for the experiment was calculated at about 100µL per well before preparation. However, slightly more than the amount required was prepared. The concentrated HRP conjugate was centrifuged at 800 x g for 1 minute, it was then diluted at 100 x concentrated HRP conjugate to 1 x working solution with HRP conjugate.

Assay preparation

1. All samples and standard to be assayed were done in duplicate
2. The wells for diluted standard, blank and sample were determined
3. One hundred micro litre (100 µL) each of the standard, blank and sample were added into the appropriate wells. The plate was covered with a sealer provided by the manufacturer. It was incubated for 90 minutes at 37ºC. (to avoid foaming as much as possible, the solution was carefully added to the bottom of the micro-ELISA plate well and touching of the inside wall was avoided).
4. The liquid from each well was decanted. Without washing, 100 µL of the biotinylated detection antibody working solution was added to each well. The plate was sealed with a new sealer and incubated for 1 hour at 37ºC.
5. The solution was again decanted from each well. 350 µL of wash buffer was added to each well. Soaked for 1 minute and aspirated the solution from each well. It was then patted to dry against a clean absorbent paper. This wash step was repeated 3 times.
6. One hundred micro litre (100 µL) of HRP conjugate working solution was added to each well. The plate was covered with a new sealer and incubated for 30 minutes at 37ºC.
7. The solution was decanted from each well, and the wash process was repeated 5 times as done in step 5 above.
8. Ninety micro litres (90 µL) of substrate reagent was added to each well. The plate was covered with a new sealer and incubated for 15 minutes at 37ºC. A foil paper was used to cover the plate to protect it from light. The plate was pre-heated for 15 minutes before optical density determination.
9. Fifty micro litres (50 µL) of stop solution was added to each well

10. The optical density (OD) of each well was determined once with microplate reader set at 450 nm.

APPENDIX 6B: PROTOCOL FOR THE DETERMINATION OF KIM-1 USING ELISA

Reagent preparation

1. All reagents were brought to room temperature before use
2. The stored serum sample was centrifuged at 1000 x g for 5 minutes
3. **Wash buffer:** Seven hundred and fifty milli-litres (750 mL) of wash buffer was prepared by diluting 30 ml of concentrated wash buffer with 720ml of deionized water. To make sure there was no crystal left, the buffer was gently warm in a 40ºC water bath and gently mixed until all the crystals dissolved.
4. **Standard working solution:** the standard was centrifuged at 10, 000 x g for 1 minute, 1 ml of reference standard and sample diluent was added and allowed to stand for 10 minutes, it was gently inverted several times. When it fully dissolved, it was thoroughly mixed with pippete. This produced a working solution of 20 ng/ml (Standard working solution).

Dilution method: seven Eppendorf tubes were used. Five hundred micro litre (500 µL) of the 20 ng/ml standard working solution was pipetted to the first tube labelled tube1 and mixed up to produce 10 pg/ml working solution. Then, 500 µL of the sample diluent were added into the remaining tubes labelled 2-7. A serial dilution was made by transferring 500 µL from tube 1 to tube 2, and from tube 2 to tube 3 up to tube 6. Each tube was thoroughly mixed before transferring to the next tube. Tube 1 served as a high standard, sample diluent in the last tube (tube 7) served as a blank, as shown in figure below.

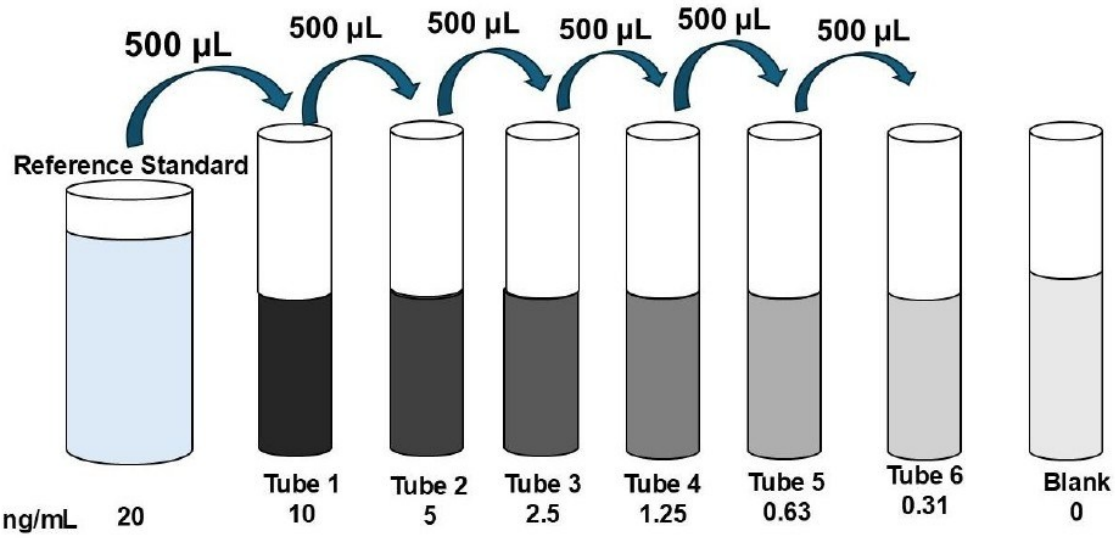

Figure : tubes preparation and dilution

The dilution gradient was kept at 20, 10, 5, 25, 1.25, 0.63, 0.31, and 0 ng/mL from the standard working solution to the blank respectively.

1. **Biotinylated detection antibody working solution**: the amount of the solution needed for the experiment was calculated at about 100µL per well before preparation. However, a slightly more than amount required was prepared. The concentrated biotinylated detection antibody was centrifuged at 800 x g for 1 minute, it was then diluted at 100 x concentrated biotinylated detection antibody to 1 x working solution with biotinylated detection antibody diluent
2. **HRP (Horseradish Peroxidase) Conjugate working solution**: the amount of the solution needed for the experiment was calculated at about 100µL per well before preparation. However, a slightly more than amount required was prepared. The concentrated HRP conjugate was centrifuged at 800 x g for 1 minute, it was then diluted at 100 x concentrated HRP conjugate to 1 x working solution with HRP conjugate.

Assay preparation

1. All samples and standard to be assayed was done in duplicate
2. The wells for diluted standard, blank and sample was determined
3. One hundred micro litre (100 µL) each of the standard, blank and sample was added into the appropriate wells. The plate was covered with a sealer provided by the manufacturer. It was incubated for 90 minutes at 37ºC. (to avoid foaming as much as possible, the solution was carefully added to the bottom of the of the micro-ELISA plate well and touching of the inside wall was avoided).
4. The liquid from each well was decanted. Without washing, 100 µL of the biotinylated detection antibody working solution was added to each well. The plate was sealed with a new sealer and incubated for 1 hour at 37ºC.
5. The solution was again decanted from each well. 350 µL of wash buffer was added to each well. Soaked for 1 minute and aspirated the solution from each well. It was then patted to dry against a clean absorbent paper. This wash step was repeated 3 times.
6. One hundred micro litre (100 µL) of HRP conjugate working solution was added to each well. The plate was covered with a new sealer and incubated for 30 minutes at 37ºC.
7. The solution was decanted from each well, and the wash process was repeated 5 times as done in step 5 above.
8. Ninety micro litres (90 µL) of substrate reagent was added to each well. The plate was covered with a new sealer and incubated for 15 minutes at 37ºC. A foil paper was used to cover the plate to protect it from light. The plate was pre-heated for 15 minutes before optical density determination.
9. Fifty micro litres (50 µL) of stop solution was added to each well

10. The optical density (OD) of each well was determined once with microplate reader set at 450 nm.