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Stopping the progression towards type 2 diabetes mellitus: investigating the hypoglycaemic (glucose-lowering) potential of antioxidant-rich plant extracts

A thesis presented in partial fulfillment of the requirements for the degree of

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Abstract

Background: Prediabetes is a condition where the blood glucose levels are high but not high enough to be classified as having type 2 diabetes mellitus (T2DM). It is also considered a high risk for developing T2DM. There is increasing evidence that demonstrates antioxidant-rich plant extracts exhibiting hypoglycaemic effects in humans. Therefore the extracts may improve glycaemic control in individuals with prediabetes and help prevent or delay the progression of prediabetes towards T2DM.

Overall Aim: To examine the acute hypoglycaemic potential of four antioxidant-rich plant extracts, namely the New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts in humans.

Methods/Design: The hypoglycaemic effects of the New Zealand pine bark was examined in healthy participants (n=25) in an acute, placebo-controlled, single-blind, crossover, dose-response (50 and 400 mg), exploratory study (Pine Bark study). Blood samples were collected via finger pricking using disposable lancet to measure glucose levels at -20, 0, 15, 30, 45, 60, 90 and 120 min during an oral glucose tolerance test (OGTT) with 75 g of glucose. The hypoglycaemic effects of grape seed, rooibos tea and olive leaf extracts matched for antioxidant capacity were examined in an acute, placebo-controlled, crossover study (GLARE study) in participants with prediabetes (n=19). Blood samples were collected via cannulating the antecubital fossa region of the arm at -10, 0, 15, 30, 45, 60, 90 and 120 min during the OGTT with 75 g of glucose. Outcome glycaemic measures were analysed in both clinical studies (Pine Bark study and GLARE study). An *in vitro* mechanistic study investigating the potential inhibitory action of all four plant extracts (grape seed, rooibos tea, olive leaf and New Zealand pine bark) on digestive enzyme α-amylase and the dipeptidyl peptidase-4 (DPP4) enzyme were carried out using appropriate enzymatic assays of inhibition.

Results: Prior to secondary analysis in the Pine Bark study, a significant reduction in the primary outcome mean glucose incremental area under the curve (iAUC) was only observed for the 400 mg dose of pine bark (21.3% reduction, p=0.016) compared to control. After stratification in the monophasic glucose curve shape group (n=12), 50 and 400 mg of pine bark significantly reduced the mean glucose iAUC compared to control (28.1% reduction, p=0.034 and 29.5% reduction, p=0.012), respectively. In contrast, mean glucose iAUC was not significantly different in the complex glucose curve shape group (n=13). In the

monophasic group, 400 mg dose further improved glycaemic indices by reducing mean percentage increment of postprandial glucose (%PG) (33.9% reduction, p=0.010), mean glucose peak (11.2% reduction, p=0.025), and mean 2h postprandial glucose (2hPG) (8.9% reduction, p=0.027) compared to control. Within the complex group, there were no other significant changes except for reductions in mean %PG after 50 mg and 400 mg dose (33.8% reduction, p=0.012 and 41.4% reduction, p=0.025) compared to control, respectively. There were no significant differences between treatments in both subgroups (p>0.05).

In the GLARE study, there were no overall significant changes in glucose and insulin responses between the extracts and control, or amongst the plant extracts (p>0.05). After secondary analysis, the less healthy subgroup (n=9), grape seed consumption showed significant reduction in mean glucose iAUC (21.9% reduction, p=0.016), mean 2hPG (14.7% reduction, p=0.034) and mean 2h postprandial insulin (2hPI) (22.4% reduction, p=0.029), whilst there was significant improvement in mean overall insulin sensitivity ($ISI_{overall}$) (15.0% increase, p=0.028) and mean glucose metabolic clearance rate (MCR) (16.7% increase, p=0.016) compared to control. Rooibos tea extract was shown to improve β-cell function measured by the mean oral disposition index (DI) (32.4% increase, p=0.031) in the less healthy subgroup compared to control. This was coupled with a non-significant improvement in insulin sensitivity measured by mean insulin-secretion-sensitivity-index-2 (ISSI-2) (18.3% increase, p=0.074). Olive leaf exhibited improved mean insulin sensitivity indices of insulinogenic index (IGI_{30}) (27.8% increase, p=0.078), Stumvoll first phase insulin sensitivity (ISI_{first}) (17.8% increase, p=0.075) and Stumvoll second phase insulin sensitivity (ISI_{second}) (15.6% increase, p=0.062) in the less healthy subgroup compared to control, although significance was not reached. Olive leaf extract was also consistently shown to elevate insulin levels in the study, with a higher mean 2hPI in the healthier subgroup (49.5% increase, p=0.030) and an elevated mean insulin iAUC in the less healthy (16.7% increase, p=0.040) subgroups. There were no significant changes in glucose and insulin responses in the healthier subgroup (n=10) compared to control nor between treatments in both subgroups (p>0.05).

The mechanistic study demonstrated that the New Zealand pine bark extract exhibited the greatest inhibitory effects against digestive enzyme α-amylase (IC_{50} 3.98 ± 0.11 mg/mL) and DPP4 enzyme (IC_{50} 2.51 ± 0.04 mg/mL) compared to the other extracts (p<0.001). Both grape seed and rooibos tea extracts showed good inhibition of both enzymes tested. Rooibos tea was able to inhibit DPP4 enzyme to a greater extent than grape seed (p=0.018). In

contrast, olive leaf extract showed minimal inhibition on α-amylase and no inhibition action against DPP4 enzyme.

Conclusions: All four plant extracts (New Zealand pine bark, grape seed, rooibos tea and olive leaf) have shown acute hypoglycaemic potential in the Pine Bark study and the GLARE study by improving various indices of glucose and insulin responses in humans. The inhibitory action of the New Zealand pine bark, grape seed and rooibos tea extracts on DPP4 enzyme might have contributed to the hypoglycaemic effects observed in the clinical studies conducted. Whereas for olive leaf extract other underlying mechanisms on glycaemia remain to be elucidated. Our acute studies have indicated the need to investigate the chronic impact of these plant extracts in longer-term studies. Future studies in the prediabetes cohort should also look to target different metabolic profiles of varying degrees of dysglycaemia, as this may provide more meaningful results.

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Abbreviations

1,5-AG	1,5-anhydroglucitol
2hPI	2h postprandial insulin
2hPG	2h postprandial glucose
%PG	Percentage increment of postprandial glucose
ACO-1	Acyl CoA oxidase-1
ADA	American Diabetes Association
ALT	Alanine aminotransferase
AI	Atherogenic index
AMPK	5' adenosine monophosphate-activated protein kinase
AST	Aspartate aminotransferase
$AUC_{glucose}$	Area under the curve of glucose
$AUC_{insulin}$	Area under the curve of insulin
BFP	Body fat percentage
BMI	Body mass index
BP	Blood pressure
BW	Body weight
CRF	Case record form
CRP	C-reactive protein
COX2	Cyclooxygenase-2 protein
CPT-1β	Carnitine palmitoyltransferase-1β
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DI	Oral disposition index
DMSO	Dimethyl sulfoxide
DNS	3,5-dinitrosalicylic acid
DNJ	1-deoxynojirimycin
DPP	Diabetes Prevention Program
DPP4 enzyme	Dipeptidyl-peptidase-4 enzyme
DPS	The Finnish Diabetes Prevention Study
DREAM	Diabetes Reduction Assessment with ramipril and rosiglitazone Medication
EDTA	Ethylenediaminetetraacetic acid

EGCG	epigallocatechin-3-gallate
EGP	Endogenous glucose production
FBG	Fasting blood glucose
FCP	Fasting C-peptide
FFA	Free fatty acid
FI	Fasting insulin
FOXO1	Forkhead box protein O1
G6Pase	Glucose-6-phosphatase
GA	Glycated albumin
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitory polypeptide
GK	Glucokinase
GLARE study	Glucose Lowering Antioxidant-Rich plant Extracts study
Glc_{max}	Glucose maximum concentration
GLP-1	Glucagon-like peptide-1
GLUT2	Sodium-independent glucose transporter-2
GMP	Good manufacturing practice
GP	General practitioner
GSP	Glycated serum protein
H-Gly-Pro-AMC	Gly-Pro-Aminomethylcoumarin
HbA1c	Glycated haemoglobin (A1c)
HDL	High-density lipoprotein cholesterol
HOMA-β	Homeostatic model assessment-beta
HOMA-IR	Homeostatic model assessment-insulin resistance
HTR	High-density lipoprotein cholesterol (HDL) to total cholesterol (TC) ratio
$iAUC_{glucose}$	Incremental area under the curve of glucose
$iAUC_{insulin}$	Incremental area under the curve of insulin
$iAUC_{sucrose}$	Incremental area under the curve of sucrose
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IFG/IGT	Combined impaired fasting glucose and impaired glucose tolerance
IGI_{30}	Insulinogenic index
IGT	Impaired glucose tolerance

IL-6	Interleukin-6
IRS2	Insulin receptor substrate 2
ISI_{first}	Stumvoll first phase insulin sensitivity index
$ISI_{overall}$	Stumvoll overall insulin sensitivity index
ISI_{second}	Stumvoll second phase insulin sensitivity index
ISI/M	Matsuda-DeFronzo insulin sensitivity index
ISSI-2	Insulin-secretion-sensitivty-index-2
LDL	Low-density lipoprotein cholesterol
K_{ATP} channel	Adenosine triphosphate-sensitive potassium channel
MCP-1	Monocyte chemoattractant protein-1
MCR	Metabolic clearance rate of glucose
MMTT	Mixed meal tolerance test
MUHEC	Massey University Human Ethics Committee
MUHNRC	Massey University Human Nutrition Research Centre
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NF-κB	nuclear factor kappaB
NGT	Normal glucose tolerance
NZ PHO	New Zealand Primary Health Organisation
NZSSD	New Zealand Society for the Study of Diabetes
OGIS	Oral glucose insulin sensitivity
OGTT	Oral glucose tolerance test
ORAC	Oxygen radical absorbance capacity
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate saline buffer
PCP	Postprandial C-peptide
PCP AUC	Area under the curve of postprandial C-peptide
PCP iAUC	Incremental area under the curve of postprandial C-peptide
PEPCK	Phosphoenolpyruvate carboxykinase
PG	Postprandial glucose
PG AUC	Area under the curve of postprandial glucose
PG iAUC	Incremental area under the curve of postprandial glucose
PI	Postprandial insulin
PI3K	Phosphoinositide 3-kinase

PI AUC	Area under the curve of postprandial insulin
PI iAUC	Incremental area under the curve of postprandial insulin
PL	Phospholipid
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
PTP1B	Protein tyrosine phosphatase 1B
p-NPG	p-nitro-phenyl- α-D-glucopyranoside
QUICKI	Quantitative insulin sensitivity check index
RCT	Randomised controlled trial
REML	Restricted maximum likelihood (statistics)
SAM	San Antonio Metabolism study
SBP	Systolic blood pressure
SEM	Standard error of the mean
SGLT1	Sodium-dependent glucose co-transporter-1
SGLT2	Sodium-dependent glucose co-transporter-2
SPARCL1	SPARC-like protein 1 precursor
STOP-NIDDM	Study to Prevent Non-Insulin-Dependent Diabetes Mellitus
SUR1	Sulfonylurea receptor-1
T2DM	Type 2 diabetes mellitus
TAC	Total antioxidant capacity
TC	Total cholesterol
TE	Trolox equivalent
TG	Triglyceride
TNF-α	Tumor necrosis factor-α
Tris-HCl	Tris hydrochloride
WC	Waist circumference
WHO	World Health Organization
WHR	Waist-hip ratio

Chapter 1
Introduction

This chapter introduces the relevance of this PhD study followed by outlining four specific research questions. The chapter will conclude with a discussion of the outline of the thesis, and contributions of each researcher involved in this work.

1.1 Background

1.1.1 Diabetes and its prevalence

It is estimated that there are currently 463 million (ages 20-79 years) (9.3%) people living with diabetes worldwide, and this number is expected to increase to an alarming 700 million (10.9%) by 2045 [1]. Last year it was estimated that about 4.2 million adults (20-79 years) died from diabetes and its complications, equivalent to one death every eight seconds [1]. Diabetes imposes a significant economic impact, with the current annual global health expenditure on diabetes estimated to be USD 760 billion and this is projected to reach USD 845 billion by 2045 [1].

Type 2 diabetes mellitus (T2DM) is a state of hyperglycaemia where glucose levels are higher than normal levels indicating insulin deficiency and pancreatic β-cell dysfunction [1, 2]. According to American Diabetes Association (ADA) and World Health Organization (WHO), T2DM is defined by fasting blood glucose (FBG) ≥7.0 mmol/L (≥126 mg/dL), or 2h postprandial glucose (2hPG) ≥11.1 mmol/L (≥200 mg/dL), or glycated haemoglobin A1c (HbA1c) ≥48 mmol/mol (≥6.5%) [1, 3].

Type 2 diabetes mellitus is the predominant form of diabetes, comprising 90 to 95% of all diagnoses [4, 5]. Although certain factors such as the environment, genetics, urbanisation, political and socioeconomic influences may determine individual susceptibility towards T2DM, poor diet choices with high levels of red meat and processed meat, refined grains, and sugary beverages, and a sedentary lifestyle also contribute to the obesity epidemic that may increase T2DM risk [6, 7]. Type 2 diabetes mellitus is independently associated with all-cause and cardiovascular disease (CVD) mortality [8]. This may be attributed to the development of macrovascular and microvascular complications from increased insulin resistance, inflammation, endothelial dysfunction, dyslipidaemia and glucotoxicity caused by elevated levels of blood glucose [9].

1.1.2 Early prevention of type 2 diabetes mellitus

Preventing T2DM brings substantial benefits, where the individual is able to avoid entering into years of drug therapy and complications [2]. Preventing T2DM has been shown to be more cost-effective than treating T2DM and its complications [10-12]. A mathematical model created by Ha and colleagues (2016) demonstrated that it is easier to prevent T2DM than to manage or cure it [13]. To minimise the health and economic burden of T2DM, it is important that strategies are found to prevent or slow down the progression from

normoglycaemia, prediabetes and into T2DM by putting a halt or delay of deteriorating glucose homeostasis [14].

The development of T2DM is often preceded by an interlude of intermediate dysglycaemia, or otherwise known as the state of prediabetes [15]. Prediabetes is an intermediate state of hyperglycaemia where blood glucose levels are elevated from normoglycaemia but they are not high enough to be classified as having T2DM [16]. Indeed, the development of hyperglycaemia is a continuum and the classification of prediabetes is to some extent an arbitrary number [17]. Research indicates that by the time individuals reach a state of impaired glycaemic control or prediabetes, their risk of mortality and morbidity, due to the development of cardiovascular and microvascular complications, has already increased [15, 16, 18, 19]. It is therefore vitally important to remedy the state of prediabetes as soon as possible, with interventions targeting the state of prediabetes as a form of early diabetes prevention treatment [20, 21].

Research has shown that reversion of high blood glucose levels to normal levels or a reduction in T2DM risk in the state of prediabetes is usually achievable when adequate lifestyle modification is in place including increasing adherence to a healthy dietary pattern and sufficient physical activity [22-24]. Large cohort studies in diabetes prevention such as The Finnish Diabetes Prevention Study (DPS) [25-27], Diabetes Prevention Program (DPP) [28-30], and Da Qing Diabetes Prevention study [31-33] have demonstrated that intensive lifestyle modification through diet and exercise interventions can reduce T2DM risk. A recent ADA consensus report summarises the current recommendations on nutrition therapy combined with physical activity for people with prediabetes [34]. Early drug therapy to prediabetes in studies such as the Study to Prevent Non-Insulin-Dependent Diabetes Mellitus (STOP-NIDDM) with acarbose [35], DPP with metformin [28], and the Diabetes Reduction Assessment with ramipril and rosiglitazone Medication (DREAM) [36] have also seen reduction in risk of T2DM development, with participants with prediabetes returning to having normal blood glucose levels. The DPP study demonstrated that lifestyle modification was more effective than drug therapy in reversing prediabetes back to normoglycaemia and delaying the onset of T2DM [28].

The DPP [37, 38] and STOP-NIDDM [39] studies showed that instigating early treatment in people with prediabetes was associated with a CVD risk reduction, which is an important clinical endpoint. However other studies including DPS [40], and the Da Qing IGT Diabetes study [31, 33] were less conclusive of lifestyle modification conferring CVD risk reduction benefits. The Whitehall II cohort study in free-living conditions recently reported

reversion from prediabetes to normoglycaemia in 37% of the participants with prediabetes based on 2hPG criterion, and was associated with a reduction of CVD risk by half (12.7 vs 29.1 per 1000 person-years, p=0.020) during five years of follow-up [41]. Early diagnosis and treatment could therefore provide an opportunity to reduce the risk of prediabetes developing into T2DM and minimise the damage caused by hyperglycaemia [22, 42, 43].

1.1.3 Antioxidant-rich plant extracts as potential hypoglycaemic agents

Antioxidant-rich plant extracts have been shown to engage multi-targeted mechanisms of action to treat hyperglycaemia with little to no adverse effects compared to anti-diabetic drugs [44-55]. This may make them ideal candidates for the therapeutic treatment of complex metabolic diseases such as prediabetes [44-55]. This has led to increasing interest in the potential of antioxidant-rich plant extracts, abundant in polyphenols, to compliment the diet and improve glycaemia, and therefore help to prevent or delay T2DM development [44-55]. Plant extracts have been demonstrated to modulate various glycaemic pathways such as glycolysis, Krebs cycle, gluconeogenesis and carbohydrate metabolism such as suppressing glucagon release, enhancing the incretin effect, delaying carbohydrate digestion and glucose absorption to maintain glucose homeostasis [45, 50, 55, 56]. The complex and unique phenolic structures of the plant extracts have been shown to be responsible for the major multifaceted role in their hypoglycaemic effects [57-62]. More details are discussed in Chapter 2.4-2.6 of the thesis. There is a growing number of human studies done to date examining the beneficial effects of a range of extracts on glucose and insulin responses in both healthy and diabetes cohorts [63, 64] compared to the prediabetes cohort [65].

1.2 Justification for the PhD study

Few studies have explored the hypoglycaemic potential of antioxidant-rich plant extracts in a prediabetes cohort. Therefore, the focus of this PhD work is to conduct nutritional intervention at the prediabetes stage, with the aim of identifying potential plant extracts that could improve glycaemic control and ultimately stop or delay the progression towards T2DM. A literature search identified four antioxidant-rich plant extracts, which are the New Zealand pine bark, grape seed, rooibos tea and olive leaf to be examined for their hypoglycaemic potential in humans. There have been no studies yet conducted on the New Zealand pine bark on glycaemic responses in humans. Plant extracts such as grape seed, rooibos tea and olive leaf have not been examined for their acute hypoglycaemic impact in

individuals with prediabetes. Therefore the primary objectives of the PhD work are to investigate the effects of these plant extracts on acute postprandial blood glucose responses in two separate human studies; Pine Bark study (investigating hypoglycaemic potential of New Zealand pine bark in healthy subjects) and GLARE study (investigating hypoglycaemic potential of extracts of grape seed, rooibos tea, and olive leaf in prediabetes cohort). The underlying mechanisms of action of the potential hypoglycaemic effects of the plant extracts will also be explored in order to understand how they work to improve glycaemic responses in humans.

1.3 Research Questions

The PhD work aims to answer four research questions arising from the study of the hypoglycaemic potential of plant extracts (**Figure 1.1**). The first research question aims at gathering evidence and having an overall review on the current studies that have examined the impact of plant extracts in improving glycaemic control in the prediabetes cohort. Research questions 2-4 are aligned to the four plant extracts: New Zealand pine bark, grape seed, rooibos tea and olive leaf being examined in this PhD study.

1.3.1 Research Question 1

Do plant extracts improve postprandial glycaemia in individuals with prediabetes?

Human clinical trials on plant extracts (years 2010-2020) and their impact on hypoglycaemic effects in individuals with prediabetes will be reviewed and summarised in a narrative review. The narrative review aims to achieve increased awareness of plant extract consumption as a potential adjunct to prediabetes management.

1.3.2 Research Question 2

Does New Zealand pine bark improve postprandial glycaemia in healthy individuals?

The New Zealand pine bark (Enzogenol®) is obtained from *Pinus radiata* trees grown commercially in New Zealand. The New Zealand pine bark has gained interest in its potential hypoglycaemic effects, partly because of its close relation to Pcynogenol®, a French pine bark, which has been reported in *in vitro* and human studies to regulate and improve markers of glucose metabolism [66-68]. To date, no clinical study examining the impact of New Zealand pine bark on glycaemic response in humans has been conducted. Therefore, an acute, placebo-controlled, single-blind, crossover, dose-response, exploratory study (Pine Bark

study) has been designed to investigate its potential hypoglycaemic effects at low and high doses during an oral glucose tolerance test (OGTT) in healthy participants.

1.3.3 Research Question 3

Do extracts of grape seed, rooibos tea and olive leaf improve postprandial glycaemia in individuals with prediabetes?

In previous work, the following plant extracts (grape seed, rooibos tea, amla berry, and green tea) have been shown to reduce postprandial blood glucose in healthy participants from between 20 to 40%, p<0.05, compared to control [69]. de Bock and colleagues (2013) conducted a 12-week study involving the consumption of olive leaf extract on overweight, middle-aged men, and they demonstrated improved insulin sensitivity and improved pancreatic function [70]. It has been proposed that the positive outcomes of grape seed, rooibos tea and olive leaf in improving glycaemic control could be translated to the prediabetes population in New Zealand. Hence, an acute, single-blind, placebo-controlled, crossover study (GLARE study) is designed to investigate whether these three plant extracts: grape seed, rooibos tea and olive leaf could improve postprandial blood glucose and insulin responses in participants with prediabetes.

1.3.4 Research Question 4

What are the underlying hypoglycaemic mechanisms of these plant extracts on improving glucose homeostasis?

The potential mechanism of action of plant extracts in controlling glucose metabolism is discussed in Chapter 2.6. The New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts have been shown to influence glucose metabolism via different underlying mechanisms. However, studies on the enzyme inhibition of α-amylase and dipeptidyl-peptidase-4 (DPP4) that are key enzymes for the regulation of postprandial glycaemia have been scarce and often subject to different assay methodologies resulting in varying study outcomes. Therefore the inhibition of the two aforementioned enzymes will be explored and their efficacy of inhibition compared amongst the extracts in order to gain insight into how New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts can improve postprandial glycaemia.

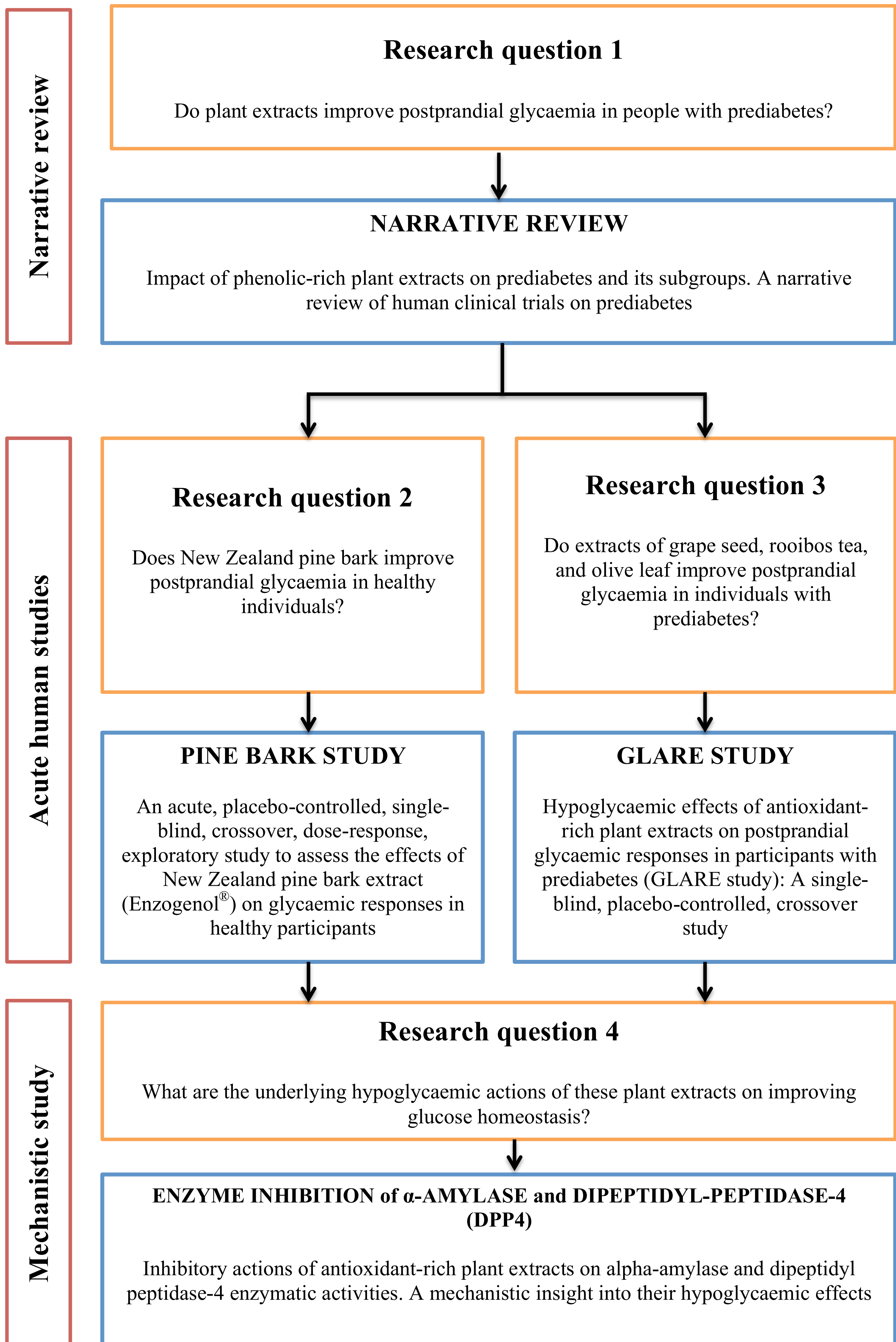

Figure 1.1 Flow diagram of the PhD research work

1.4 Thesis structure

This thesis follows the format of a PhD by publication. The thesis chapters are outlined below with identification of those chapters representing materials that have been published, submitted, or ready for submission for publication.

Chapter 1 introduced the aims and objectives of the PhD study summarised into four research questions to be answered. It also provides the rationale and importance of the work presented in this thesis in the area of exploring nutritional alternatives for the prediabetes cohort in order to prevent or slow prediabetes progression to T2DM.

Chapter 2 presents an overview of what prediabetes is and the criteria for diagnosis, followed by the discussion on the important role of antioxidant-rich plant extracts in glycaemic control. Evidence from *in vitro* and *in vivo* studies including human clinical trials involving plant extracts focusing on four identified plant extracts, namely the New Zealand pine bark, grape seed, rooibos tea and olive leaf, and their impact on glycaemic control, are extensively covered in this chapter. Research investigating the hypoglycaemic mechanisms of action of these extracts will also be discussed.

Chapter 3 is an extension of the literature review and addresses Research Question 1. This chapter focuses on gathering clinical evidence on the hypoglycaemic impact of plant extracts in the prediabetes cohort. This chapter has been prepared as a narrative review for submission to the *Critical Reviews in Food Science and Nutrition* Journal.

Chapter 4 presents the first results chapter and focuses on the hypoglycaemic potential of the New Zealand pine bark in healthy participants and addresses Research Question 2. This chapter is a published manuscript in *Nutrients* Journal on the Pine Bark study regarding the impact of the New Zealand pine bark (Enzogenol®) in healthy participants.

Chapter 5 is the second results chapter examining the hypoglycaemic potential of three plant extracts: grape seed, rooibos tea and olive leaf in participants with prediabetes in the GLARE study and addresses Research Question 3. This study has been prepared as a manuscript for submission to *The Journal of Nutrition*.

Chapter 6 is the third results chapter focusing on determining some of the underlying mechanistic actions of New Zealand pine bark, grape seed, rooibos, and olive leaf extracts on glycaemic control and addresses Research Question 4. This data has been prepared as a short communication manuscript for the *Nutrients* Journal.

Chapter 7 is the overall discussion and conclusion of the PhD study highlighting the main findings of the research. The strengths and limitations of the research and recommendations for future studies on prediabetes are discussed.

The Appendices chapter contains the research outputs associated with this PhD study and other study protocol diagrams, templates of participant information sheet and consent forms of the Pine Bark study and the GLARE study, as well as other relevant information pertaining to the clinical trials.

1.5 Researchers' Contribution

Table 1.1 outlines the contribution of researchers involved in this PhD study.

Table 1.1 Contributions by each researcher involved in this PhD study

Researchers	Contribution
Wen Xin Janice Lim **PhD researcher**	Responsible for all aspects of the two human clinical trials (Pine Bark study and the GLARE study) involving study design, human ethics application, participant recruitment, data collection, statistical analysis and interpretation of data Responsible for all aspects of the mechanistic study on the enzyme inhibition of plant extracts including sample preparation to be analysed by external lab (Callaghan Innovation), statistical analysis and interpretation of data Responsible for all aspects of the manuscripts involving conceptualisation and design of manuscripts, literature search, data extraction and analysis, drafting, editing and submission of manuscripts

Assoc Prof Rachel A. Page **Primary supervisor**	Conceptualisation and design of the Pine Bark study and the GLARE study, acquisition of funding and human ethics approval, supervision of trials and reviewing of thesis and all manuscripts
Dr Cheryl S. Gammon **Co-supervisor**	Conceptualisation and design of the Pine Bark study and the GLARE study, supervision of trials, reviewing of thesis and all manuscripts
Assoc Prof Pamela R von Hurst **Co-supervisor**	Conceptualisation and design of the Pine Bark study and the GLARE study, acquisition of funding, supervision of trials and reviewing of thesis and all manuscripts
Lynne Chepulis **External Co-supervisor**	Conceptualisation and design of the Pine Bark study and the GLARE study, acquisition of funding, supervision of trials and reviewing of thesis and all manuscripts
Owen Mugridge **Research Trials Manager**	Participant recruitment and management, data collection, and a phlebotomist for GLARE participants

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Chapter 2

Review of the literature

This chapter begins with an overview of prediabetes and its diagnosis criteria, followed by discussing the important role of antioxidant-rich plant extracts in glycaemic control, with special attention given to the New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts, which are plant extracts that will be included in the two human clinical trials in this PhD study. The hypoglycaemic mechanisms of action of the plant extracts are also discussed.

2.1 What is prediabetes?

In normal healthy individuals blood glucose is strictly regulated to maintain glucose concentration between 3.9 and 5.6 mmol/L [5]. After a meal glucose is absorbed and blood glucose levels begin to increase. A rise in blood glucose stimulates insulin secretion that facilitates glucose uptake into the cells and blood glucose level returns to normal blood glucose concentration range after 1-3 h [7].

However in prediabetes, the glucose homeostasis becomes increasingly disturbed, which leads to uncontrolled higher levels of blood glucose or an intermediate state of hyperglycaemia but not high enough to be diagnosed with T2DM [9]. As hyperglycaemia persists, prolonged excessive glucose in the bloodstream may start to induce oxidative stress that promotes the development of insulin resistance [5, 10-12]. However, with increasing insulin resistance individuals may still remain glucose tolerant if their pancreas is able to compensate with hypersecretion of insulin [13]. Nonetheless, as damage to β-cell function continues, the compensatory hyper-secretion of insulin is lost and T2DM gradually develops, giving rise to complications such as nerve damage (neuropathy), kidney damage (nephropathy) and eye disease (retinopathy, visual loss or blindness) [14, 15] and increasing the risk of developing cardiovascular disease (CVD) and hypertension [13, 16].

2.2 Prediabetes as a global health issue

Individuals with prediabetes have a high risk for developing T2DM [6, 17, 18], with an annual conversion rate of 5-10% into T2DM [9, 19]. Eventually up to 70% of the people with prediabetes will develop T2DM [20]. Nonetheless, a recent statement by the American Diabetes Association (ADA) showed that an estimated two-thirds of people with prediabetes do not progress into T2DM, even after many years. Furthermore, approximately one-third of people with prediabetes return to normoglycaemia [21]. More research is required to understand the prevalence and pathogenesis of prediabetes developing into T2DM. Essentially, there exists a proportion of individuals with prediabetes progressing into T2DM if their suboptimal glycaemic condition is not managed, and hence is be an important area of research.

Globally, 373.9 million adults (20-79 years) (7.5%) have been estimated to have impaired glucose tolerance (IGT) in 2019 [15]. This has been projected to increase to 548.4 million (8.6%) by 2045. The regional prevalence is highest in North America and Caribbean (55.5 million, 12.3%), followed by Western Pacific (136.5 million, 10.4%), and Africa (45.3

million, 10.1%) [15]. With respect to countries, the highest prevalence of IGT is in China (54.5 million), followed by the United States of America (37.4 million), Indonesia (29.1 million), and India (25.2 million) [15]. It is noteworthy that the estimates were based only on IGT measures, the most studied prediabetes subgroup.

2.3 Prediabetes in New Zealand

In New Zealand there are few statistics on the prevalence of prediabetes. A 2015 Ministry of Health report estimated that 257,700 people have diabetes (6%), and that diabetes prevalence had been rising on average 7% annually for the past eight years [22]. The 2008/09 adult nutrition survey reported that approximately 7% of the population as having diabetes and 25.5% of the population as having prediabetes [23]. However, the data from this survey is now quite old [23].

2.3.1 Ethnicities in New Zealand and prediabetes

The prevalence of both prediabetes and T2DM in New Zealand are higher in certain population groups. It has been estimated that 40% of people of Māori, Pacific and Indian ethnicity living in the Auckland metro region have prediabetes at 35-39 years and over 50% have prediabetes at 45-49 years of age [24]. Māori and Pacific people have been shown to have two to fourfold rates of T2DM as European New Zealanders [25]. Those with T2DM also tend to have poorer health outcomes such as poorer blood glucose, blood pressure and blood lipid control, leading to higher rates of diabetic complications such as nephropathy [25]. Robinson and colleagues (2006) highlighted that risk factors such as smoking, an HbA1c > 8% (64 mmol/mol), and having microalbuminuria in the Māori and Pacific community might have contributed to the development of diabetic complications [26]. Therefore more research and investment of resources should target the Māori and Pacific group, particularly with the involvement of family support being a vital part, as well as providing trustworthy sources of dietary information to more effectively manage prediabetes and T2DM in this group [27].

2.3.2 Work in progress in New Zealand to improve glycaemic outcomes

In recent decades prediabetes has been recognised as a stage vital for early intervention to prevent or delay T2DM development [28, 29]. In New Zealand, various initiatives by the government have been laid out. These include the Green Prescriptions and Healthy Families NZ programmes with a unique community approach to promote physical

activity and improve nutrition, as well as funding the Healthier Lives National Science Challenge to conduct research on slowing or halting the progression of T2DM [22]. These initiatives are aimed at increasing current levels of health literacy, support healthy living, and provide preventive measures against T2DM development. The Green Prescriptions has since received positive behavioural changes and improved health outcomes in participants who have taken part in the programme [30-34].

2.4 Who may have prediabetes?

Table 2.1 shows the diagnostic criteria for impaired glycaemic control based on established criteria by the World Health Organization (WHO) [15, 35, 36], the American Diabetes Association (ADA) [37, 38], and The New Zealand Society for the Study of Diabetes (NZSSD) [39]. The glycated haemoglobin A1c (HbA1c) is the recommended diagnostic screening test in New Zealand to identify individuals with prediabetes (HbA1c 41-49 mmol/mol) in both clinical and research settings. Different countries may follow slightly different criteria for the identification of prediabetes, with for example the ADA using lower fasting blood glucose (FBG) of 5.6-6.9 mmol/L (100-125 mg/dL) and HbA1c level of 39-47 mmol/mol (5.7-6.4%).

Table 2.1 Diagnostic criteria for impaired glycaemic control and T2DM

Blood test	Unit	Normal	IFG	IGT	IFG/IGT	T2DM[†]
World Health Organization (1999)						
Fasting blood glucose	mmol/L	<6.1	6.1-6.9	<7.0	6.1-6.9	≥7.0
	mg/dL	<110	110-125	<126	110-125	≥126
		AND				**OR**
2h postprandial glucose*	mmol/L	<7.8	If measured, <7.8	7.8-11.0	7.8-11.0	≥11.1
	mg/dL	<140	<140	140-199	140-199	≥200
		NA				**OR**
HbA1c	mmol/mol	Currently HbA1c is not considered a suitable diagnostic test for intermediate hyperglycaemia				≥48[‡]
	%					≥6.5[‡]
American Diabetes Association (2010)						
Fasting blood glucose	mmol/L	<5.6	5.6-6.9	<5.6	5.6-6.9	≥7.0
	mg/dL	<100	100-125	<100	100-125	≥126
		AND				**OR**
2h postprandial glucose*	mmol/L	<7.8	<11.1	7.8-11.0	7.8-11.0	≥11.1
	mg/dL	<140	<200	140-199	140-199	≥200
		OR				
HbA1c	mmol/mol	<39	39-47			≥48
	%	<5.7	5.7-6.4			≥6.5
New Zealand Society for the Study of Diabetes (2012)						
Fasting blood glucose	Recommended only if HbA1c measurement is not possible					
	mmol/L	≤6.0	6.1-6.9			≥7.0
	mg/dL	≤110	110-125			≥126
		AND				**NA**
2h postprandial glucose*	Recommended only if results from HbA1c is inconclusive for specific patients (e.g. presence of haemoglobinopathy or abnormal red cell turnover). Take fasting blood glucose prior to requesting an oral glucose tolerance test to confirm.					
	mmol/L	<7.8	7.8-11.0			≥11.1
	mg/dL	<140	140-199			≥200
		OR				
HbA1c	mmol/mol	≤40	41-49			≥50
	%	≤5.8	5.9-6.6			≥6.7

* 2h postprandial glucose is determined by a standard 2h oral glucose tolerance test (OGTT) with 75 g of carbohydrates. [†] A random plasma glucose ≥11.1 mmol/L (≥200 mg/dL) alone may be used to diagnose type 2 diabetes mellitus (T2DM) according to American Diabetes Association (ADA) and The New Zealand Society for the Study of Diabetes NZSSD guidelines. [‡] A glycated haemoglobin A1c (HbA1c) value of less than 6.5% does not exclude diabetes diagnosed using glucose tests. NA: not applicable.

Glycated haemoglobin A1c was introduced in NZ as diagnostic criteria for diabetes and prediabetes in 2012. Prior to that, IFG and IGT were the measurements used for diagnosis of T2DM and impaired glycaemic control. The impaired glycaemia measured by

IFG, IGT and combined IFG/IGT represent different glycaemic pathophysiological abnormalities [40, 41]. Individuals with IGT have more muscle insulin resistance and mild hepatic insulin resistance, in contrast to those with IFG having increased hepatic insulin resistance but near normal muscle insulin sensitivity [42, 43] (**Table 2.2**). The combination of IFG and IGT marks a more advanced disturbance of glycaemic homeostasis with the inclusion of glycaemic abnormalities observed in both IFG and IGT [44-48].

Table 2.2 Pathophysiological differences between IFG and IGT

Differences between IFG and IGT	
IFG	**IGT**
Increased hepatic insulin resistance	Increased muscle insulin resistance
Normal or near to normal muscle insulin sensitivity	Normal or near to normal hepatic insulin sensitivity
Elevated fasting blood glucose	Normal or near to normal fasting blood glucose
Reduced early-phase insulin secretion during oral glucose tolerance test (0-30 min)	Reduced early-phase insulin secretion during oral glucose tolerance test (0-30 min)
Normal or near to normal late-phase insulin secretion during oral glucose tolerance test (60-120 min)	Impaired late-phase insulin secretion during oral glucose tolerance test (60-120 min)
Normal or near to normal fall in postprandial blood glucose	Sustained rise in postprandial blood glucose

Adapted from [42, 43].

Screening individuals for prediabetes is important as it provides the opportunity to begin early treatment. **Table 2.3** shows the New Zealand Guidelines for screening of individuals at risk of diabetes. Having certain risk factors can put individuals at a higher risk of developing T2DM and so it is important that they should be screened to detect for the presence of impaired glycaemic control.

Table 2.3 New Zealand Guidelines for screening of individuals at risk of diabetes

1. Adults over 25 years of age who has one or more of the following risk factors:
• Have known ischaemic heart (angina or myocardial infarction, cerebrovascular or peripheral vascular disease) • Are on long-term steroid or antipsychotic treatment • Are obese (body mass index (BMI) ≥30 kg/m^2, or ≥27 kg/m^2 in Indo-Asian*) • Have a family history of early age onset T2DM in more than one first-degree relative • Are women with past history of gestational diabetes mellitus (GDM) • Women with polycystic ovary syndrome
2. Obese children and young adults (BMI ≥30 kg/m^2, or ≥27 kg/m^2 in Indo-Asian) who has one or more of the following risk factors:
• Have a family history of early age onset T2DM, e.g. < 40 years • Are of Maori, Pacific or Indo-Asian* ethnicity

*Indo-Asian includes Indian, Fijian Indian, Sri Lankan, Afghani, Bangladeshi, Nepalese, Pakistani, and Tibetan. Adapted from [39, 49].

2.4.1 Who else may have an increased risk of developing T2DM?

Both healthy, normoglycaemic individuals as well as individuals with prediabetes may eventually develop T2DM [19, 50-53] depending on their existing patterns of postprandial glucose shapes [54-60], postprandial glucose measures [55, 61-65], and patterns of insulin levels [66-68] indicating the varying degrees of risk towards T2DM. This is because the progress from normoglycaemia towards hyperglycaemia is often a heterogeneous continuum [19, 41, 50, 52, 69, 70], and responses to intervention may thus differ depending on individual metabolic profiles.

Research is still uncovering the possible reasons why different metabolic profiles, for example, different postprandial glucose curve shapes (monophasic, biphasic and triphasic), could elucidate alterations in glucose metabolism. In healthy, normoglycaemic individuals, who are also more likely to exhibit biphasic and triphasic glucose curve shapes (also known as complex shapes), postprandial glucose tends to follow an oscillating pattern that is synchronised with the oscillations of insulin responses, suppression of endogenous glucose production, as well as insulin-stimulated glucose disposal and absorption that is influenced by rate of gastric emptying [54, 71]. Studies have elucidated that early insulin responses associated with insulin hypersensitivity may play a part in biphasic glucose curve shapes [58, 60]. Furthermore, Kaga and colleagues (2020) demonstrated that better insulin clearance in biphasic glucose curve shapes might have led to a second rise in glucose levels after reaching

nadir in order to prevent hypoglycaemia, signifying higher muscle insulin sensitivity [56]. This observation was in agreement with other researchers who explained that internal body impulse responses of the hepatic and renal endogenous glucose releases, also named endogenous excitations, were present to regulate the dynamic behaviour of blood glucose concentrations during an OGTT until blood level returned to basal value [59].

It has been suggested that it might be due to the over-compensation of insulin secretion during meal times, however due to insulin resistance in individuals with monophasic glucose curve shapes, the blood glucose remains higher than normal (hence only having one glucose peak) [57]. Other factors such as impairment in the ultradian and circadian oscillation cycles, as well as depressed pulsatility of insulin secretion in the insulin-glucose regulation system serving as an indication of reduced β-cell function that may also give rise to the monophasic shape [57]. Kim and colleagues (2016) postulated that the inadequate suppression of glucagon secretion resulting in higher endogenous glucose production would have contributed to the monophasic shape, whilst incretin and pancreatic hormones had no significant impact [55]. Due to the sub-optimal insulin and glucagon responses leading to higher postprandial glucose in monophasic shapes, it is undoubtedly indicative of increased risk for development of T2DM [54-59], and therefore an area of research for interventions.

Therefore, due to this difference in pathophysiology of glycaemic metabolic profiles in each individual, it is likely that individuals with different metabolic profiles will respond differently to a given intervention. More recent human studies looking at interventions with food products/ plant extracts have begun stratifying participants based on their degree of glucose metabolic profiles to achieve better intervention outcome in glucose responses [72-74]. Therefore, stratification of participants into their respective subgroups based on their glycaemic patterns and responses were therefore carried out as a form of secondary exploratory analysis in both human clinical trials performed in this PhD study: Pine Bark study (Chapter 4) and the GLARE: Glucose Lowering Antioxidant-Rich plant Extracts study (Chapter 5) to determine effectiveness of intervention. The narrative review (Chapter 3) will also elucidate the relevance of subgrouping based on different metabolic profiles of individuals for a more targeted treatment to improve glycaemic control.

2.5 Plant extract polyphenols and their health benefits

2.5.1 What are plant polyphenols?

Plant polyphenols are divided into flavonoids that include anthocyanidins, and several classes of non-flavonoids: phenolic acids, stilbenes, and lignans [3]. Over 8000 polyphenols have been identified and are present in fruit and beverages such as fruit juice, wine, tea, coffee, chocolate and beer, and to a lesser extent vegetables, dry legumes, and cereals [75, 76]. A typical total intake could amount up to 1 g/day [77]. Polyphenols are ubiquitously present in plant foods, thus partly explaining the good health effects of consuming vegetables and fruits or polyphenol-rich foods that are characteristic of a Mediterranean diet [78-81].

2.5.2 Hypoglycaemic potential of plant extracts on human health

Phenolic compounds from antioxidant-rich dietary sources such as edible plant extracts have attracted a great deal of attention for the past two decades due to the increasing evidence regarding their beneficial effect on human health in the prevention of metabolic diseases such as diabetes, cardiovascular diseases, neurodegenerative diseases and cancer [82-85]. Extracts from plant sources may be taken from different parts of the plant such as the root, stem, leaf, flower or fruit [86].

Consumers are also increasingly becoming more receptive towards the use of natural health products derived from plants containing high levels of polyphenols for the treatment of chronic health conditions including T2DM [87]. A 2018 comprehensive dose-response meta-analyses and systematic review of 18 prospective cohort studies has corroborated that higher amounts of polyphenol intake is associated with the lowest risk of T2DM [84]. The study concluded that an inverse association existed between polyphenols such as flavonoids, flavonols, flavan-3-ols, catechins, anthocyanidins, isoflavones, daizdzein, genistein, and stilbenes and T2DM [84].

Meta-analyses and systematic reviews of commonly consumed phenolic-rich plant sources such as teas, coffees and chocolates have revealed the importance of a diet rich in polyphenols in managing T2DM risk [88-91]. Study outcomes showed that drinking more than 3 cups of tea a day has been associated with a reduction in T2DM risk [88, 89]. Similarly, every cup-per-day increase in coffee consumption was associated with a 6% reduction in T2DM risk [90]. However, there is much less certainty regarding chocolate consumption and reduction in T2DM risk [91].

To further elaborate on a few examples of extensively studied plant extracts and their impact on glycaemic control and T2DM, green tea has been one of the more extensively studied plant-derived dietary sources of polyphenols. The high concentrations of flavonols such as epigallocatechin-3-gallate (EGCG) might have contributed to the health-promoting effects of green tea on diabetes, including obesity and CVD [92]. Meta-analysis conducted by Liu and co-workers (2013) examining 17 chronic randomised controlled trials (RCTs) in healthy, obese participants, those with T2DM, and only with two studies on prediabetes, concluded that green tea was able to improve glycaemic control and insulin sensitivity with significant reductions in FBG, fasting insulin (FI) and HbA1c [93]. A meta-analysis conducted by Zheng and colleagues (2013) examining the impact of green tea consumption in 22 chronic RCTs in participants who were healthy, obese, having metabolic syndrome or T2DM, and only one study on prediabetes showed FBG improvement as well [94]. However, Wang and colleagues (2014) in a meta-analysis of seven chronic RCTs showed no significant improvement in glycaemic parameters such as FBG, FI, 2hPG, HbA1c and homeostatic model assessment: insulin resistance (HOMA-IR) in populations at risk of T2DM with prolonged green tea consumption [95].

In other studies coffee and coffee polyphenol extract have also been shown to increase insulin secretion, raise GLP-1 response, and improve glycaemic control in humans, of which chlorogenic acid is a major component of coffee [96-99].

Cinnamon, which is a widely available and utilised spice, has also been increasingly known for its hypoglycaemic effects, which have been largely attributed to its active component cinnamaldehyde [100, 101]. A recent meta-analysis consisting of 16 RCTs on cinnamon elucidated favourable changes on FBG and HOMA-IR in participants with prediabetes but mostly were with T2DM [102]. Another spice derivative, curcumin, a phenolic compound found in turmeric, has been shown to have glucose-lowering and insulin sensitising effects for T2DM treatment [103, 104].

However to date, most of the human studies investigating the impact of phenolic-rich plant sources on improving glycaemic control have largely focused on healthy or obese participants, and mostly on the T2DM and metabolic syndrome cohorts.

2.5.3 Hypoglycaemic potential of plant extracts on prediabetes

There have been fewer human studies done to examine the impact of antioxidant-rich plant sources on prediabetes compared to T2DM. Longer-term, chronic studies on glucose and insulin responses have been conducted to date with spices [73, 105, 106], seeds [107], tea

beverages [108-110], pure bioactive components from plants [74, 111], fruits and fruit components [112-115], plants and their parts [116, 117], combined mixture of polyphenols, plant extracts and other nutrients [118-124], and traditional Chinese medicine [125, 126]. Chapter 3 is a narrative review that focuses on RCTs performed in the prediabetes cohort examining hypoglycaemic impact of plant extracts more similar in nature to the extracts examined in this PhD study.

A clear benefit of using antioxidant-rich plants or plant extracts to improve glycaemic control is there is minimal to no adverse effects as observed with anti-diabetic drug therapy [1, 127]. **Figure 2.1** shows how anti-diabetic drugs treat hyperglycaemia via different modes of actions in the body but are associated with various adverse effects. Currently there is a lack of clinical evidence regarding the chronic benefits of plant extracts on glycaemic control, including gaps in our understanding regarding potential drug-herb interactions, and interaction with food proteins during consumption would influence the efficacy and pharmacokinetics of the plant extracts on glucose metabolism [6, 75, 83, 128-130].

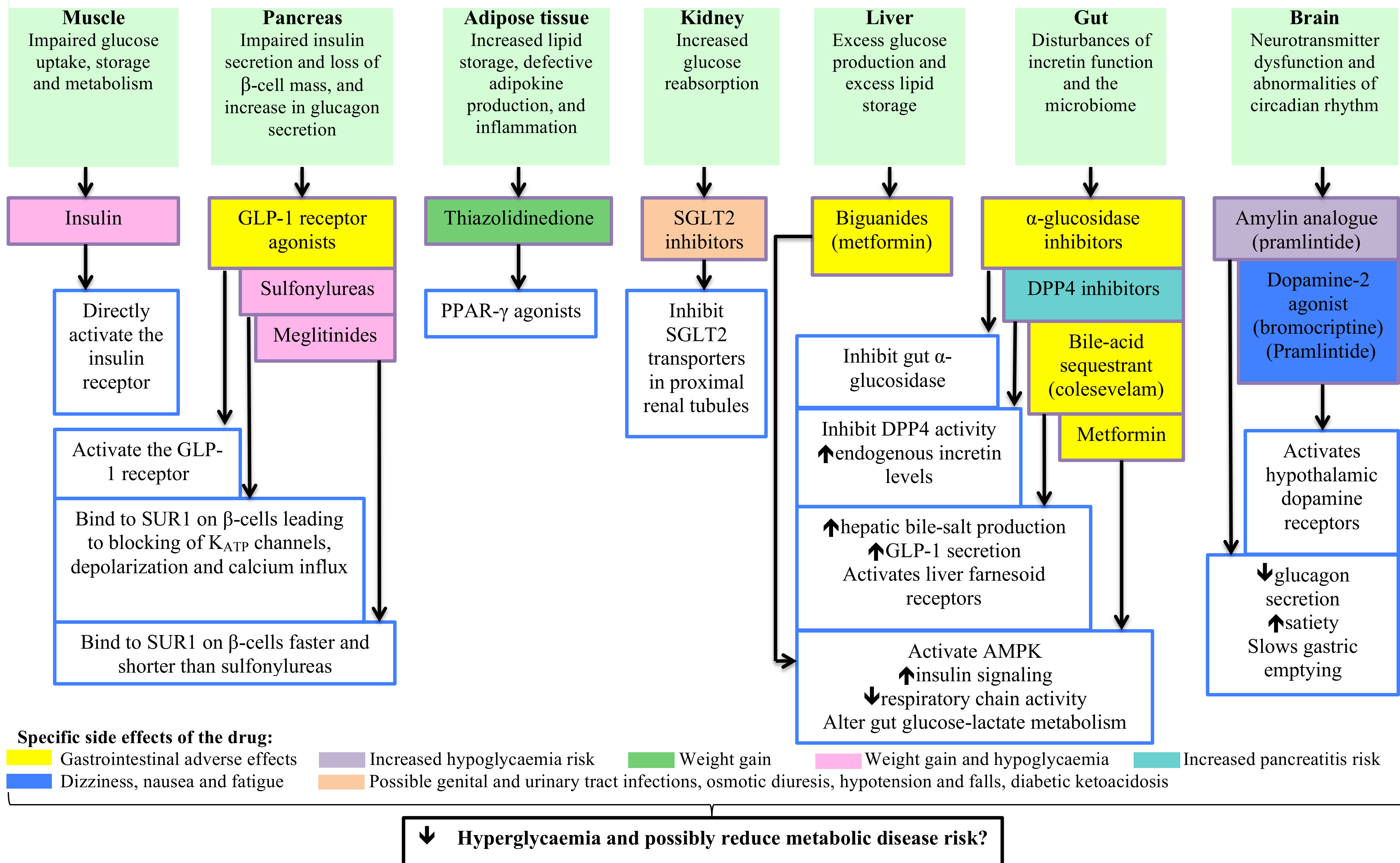


Figure 2.1 Sites of action of anti-diabetic drugs and their associated potential adverse effects

2.6 The plant extracts examined in the PhD study

Even though there have been many animal and human studies investigating the hypoglycaemic potential of plant extracts [131], the aim of this PhD study was to identify suitable plant extracts easily accessible in New Zealand that have not been investigated for their hypoglycaemic potential in the prediabetes cohort. Therefore, four plant extracts, namely the New Zealand pine bark, grape seed, rooibos tea, and olive leaf, have been selected to examine their impact on glycaemic control in humans. To date, no studies have been done to investigate the impact of the New Zealand pine bark on glycaemic control. There have also been no prior studies conducted examining acute glycaemic responses on individuals with prediabetes for grape seed, rooibos tea and olive leaf extracts. The GLARE study (Chapter 5) is a continuation of the previous work conducted by this research group that grape seed and rooibos tea significantly reduced postprandial blood glucose in healthy participants compared to control [132]. de Bock and colleagues (2013) also demonstrated improved insulin sensitivity and improved pancreatic function after 12 weeks of olive leaf consumption in cohort of obese men [133]. It is therefore hypothesised that grape seed, rooibos tea and olive leaf may have the potential to also improve glycaemic control in individuals with prediabetes.

This section provides the background of all four extracts for the designing of the human clinical trials: Pine Bark study and the GLARE study. Most of the studies are chronic studies spanning one week to six months. Improvements observed in both the acute and chronic studies were in the outcome measurements of glucose metabolism, such as HbA1c, FBG, FI, and insulin sensitivity. Some studies also showed improvements in inflammation and oxidative stress [133, 134]. The studies were mainly conducted on individuals who were healthy, diagnosed with T2DM, or having metabolic syndrome risk factors, obese or overweight (**Table 2.4**).

As whole plant extracts may elucidate more hypoglycaemic effects than their individual bioactive fractions [135-144], the extracts investigated in the PhD work, namely the New Zealand pine bark, grape seed, rooibos tea and olive leaf were whole extracts comprising a range of naturally occurring phenolic compounds.

Table 2.4 Human clinical studies to date that have investigated the hypoglycaemic effects of the plant extracts examined in the PhD study

Plant extract	Study (Type, duration)	Dosage	Participants	Significant hypoglycaemic outcome
Pine bark extract				
Pine bark extract (Pycnogenol, French maritime pine bark) [145]	RCT, double-blind, placebo-controlled, parallel study, 8 weeks. A further 60 days for open design study (n=20)	300 mg/day	Venous insufficiency, n=40 (n=10 placebo, n=30 pycnogenol)	No significant change in FBG
Pine bark extract (Pycnogenol, French maritime pine bark) [146]	RCT, double-blind, placebo-controlled, parallel study, 8 weeks. A further 60 days for open design study (n=20)	150 mg/day	Vascular retinopathy, n=40 (n=10 placebo, n=30 pycnogenol)	↓FBG
Pine bark extract (New Zealand pine bark, *Pinus radiata*) with added vitamin C [147]	Open-labelled, uncontrolled, pilot study, 12 weeks	480 mg of flavonoid extract and 240 mg vitamin C/day	Healthy, n=24	No significant change in FBG
Pine bark extract (Pycnogenol, French maritime pine bark) [148]	RCT, double-blind, placebo-controlled, parallel, multi-centre study, 12 weeks	100 mg/day	T2DM, n=77 (n=34 treatment, n=43 placebo)	↓FBG ↓HbA1c (first month only)
Pine bark extract (Pycnogenol, French maritime pine bark) [149]	Open, controlled, dose-response, crossover study, 12 weeks (each dose for 3 weeks)	50, 100, 200, 300 mg/day	T2DM, n=30	↓FBG, but 300 mg no greater effect ↓2hPG, but 300 mg no greater effect ↓HbA1c at 9 and 12 week No significant changes in insulin levels
Pine bark extract (Pycnogenol, French maritime pine bark) [150]	Controlled, parallel study, 4 weeks	150 mg/day	Severe diabetic microangiopathy, n=30 (n=16 placebo, n=14 Pycnogenol)	No significant change in FBG and HbA1c
Pine bark extract (Pycnogenol, French maritime pine bark) [151]	RCT, double-blind, placebo-controlled and active drug, parallel study, 2 weeks	180 mg/day	Healthy, n=16 (n=8 in each group)	No significant change in FBG
Pine bark extract (Pycnogenol, French maritime pine bark) [152]	RCT, double-blind, placebo-controlled, parallel study, 12 weeks	125 mg/day	T2DM and mild to moderate hypertensive, n=48 (n=24 in each group)	↓FBG and HbA1c

Pine bark extract (Pycnogenol, French maritime pine bark) [153]	Double-blind, placebo-controlled, matched-pair design study, 12 weeks	150 mg/day	Healthy elderly, n=101	No significant change in FBG
Pine bark extract (Flavagenol) [154]	RCT, double-blind, placebo-controlled, parallel study, 12 weeks	200 mg/day	Overweight and obese, n=130 (n=64 treatment, n=66 placebo)	No significant changes in insulin and FBG
Pine bark extract (Pycnogenol, French maritime pine bark) [155]	RCT, double-blind, placebo-controlled, crossover study, 8 weeks	200 mg/day	Coronary heart disease, n=23	No significant change in FBG
Pine bark extract (Pycnogenol, French maritime pine bark) [156]	Open, controlled, parallel study, 6 months	150 mg/day	Metabolic syndrome, n=130 (n=64 treatment, n=66 placebo)	🡻FBG
Pine bark extract (Pycnogenol, French maritime pine bark) [157]	Controlled, parallel study, 8 weeks	100 mg/day	Peri-menopausal women with CVD risk factors, n=70 (n=35 in each group)	🡻FBG
Grape seed extract				
Grape seed extract [158]	RCT, single-blind, placebo-controlled, parallel study, 12 weeks	200 mg/day or 400 mg/day (total procyanidins in grape seed extract)	Healthy, n=53 (n=18 in placebo and 200 mg/day, n=17 for 400 mg/day)	No significant change in FBG and HbA1c
Grape seed extract [159]	RCT, placebo-controlled, crossover study, 4 weeks	600 mg/day	High-risk CVD with T2DM, n=32	🡻Fructosamine No significant changes in FBG No significant change in HOMA-IR
Grape seed extract [160]	RCT, double-blind, placebo-controlled, parallel study, 4 weeks	150 mg or 300 mg/day (Meganatural BP)	Metabolic syndrome, n=27 (n=9 in each group)	No significant change in FBG and FI
Grape seed extract [161]	RCT, double-blind, placebo-controlled, crossover study, 4 weeks	1300 mg/day (Nature's Pearl muscadine grape seed, *Vitis rotundifolia*)	Metabolic syndrome, n=50	No significant change in FBG
Grape seed extract [162]	RCT, double-blind, placebo-controlled, parallel study, 8 weeks	200 mg/day	T2DM, n=48 (n=26 treatment, n=22 placebo)	No significant change in FBG and HbA1c
Grape seed extract [163]	RCT, double-blind, placebo-controlled, parallel study, 8 weeks	300 mg/day (Meganatural BP)	Pre-hypertensive, n=32 (n=16 in each group)	No significant change in FBG
Grape seed extract [164]	RCT, placebo-controlled, crossover, acute study, 6h OGTT (high fat-carbohydrate meal, 670 kcal)	300 mg	Metabolic syndrome, n=12	🡻iAUC glucose No significant change in AUC insulin

Grape seed extract [165]	RCT, controlled, crossover, acute study, 2h OGTT (high carbohydrate meal, 92 g carbohydrates)	100, 300 mg	Healthy, n=8	🡣2hPG 🡣iAUC glucose and AUC glucose
Grape seed extract beverage [166]	RCT, placebo-controlled study, parallel, 6 weeks, 4 week follow-up	300 mg/day (juice)	Pre-hypertensive, n=29 (n=17 in placebo, n=12 in grape seed extract)	No significant change in FI and HOMA-IR No significant change in FBG
Grape seed extract [132]	RCT, crossover, acute study, 2h OGTT (Study 1: oral glucose, 50 g carbohydrates; study 2: simple meal with white bread and ham, 50 g carbohydrates)	500 mg	Healthy, n=10	🡣iAUC glucose
Rooibos tea extract				
Rooibos tea extract [167]	Non-randomised, controlled, crossover study, 6 weeks	6 cups of rooibos tea/day (1 cup constitutes 1 tea bag (Rooibos Ltd.) with 200 mL boiled water)	Healthy but with at least two or more CVD risk factors, n=40	No significant change in FBG
Rooibos tea extract [132]	RCT, controlled, crossover, acute study, 2h OGTT (Study 1: oral glucose with 50 g carbohydrates; study 2: simple meal with white bread and ham, 50 g carbohydrates)	760 mg	Healthy, n=10	🡣iAUC glucose
Olive leaf extract				
Olive leaf extract [168]	Exploratory, crossover, acute study, 3h OGTT (300g of cooked rice)	1000 mg olive leaves	Healthy, n=7, borderline diabetic, n=7 (FBG: 6.1-7.8 mmol/L)	🡣PG at 30min and 1h (p<0.05) in borderline diabetic participants No significant changes in healthy, normoglycaemic participants
Olive leaf extract [169]	RCT, open, placebo-controlled, two-arm parallel, co-twin study, 8 weeks	500 mg/day or 1000 mg/day (EFLA 943)	Borderline hypertensive monozygotic twins, n=40 (n=10 in each group)	No significant change in FBG
Olive leaf extract [170]	RCT, placebo-controlled, parallel study, 14 weeks	500 mg/day	T2DM, n=79 (n=41 treatment, n=38 placebo)	🡣HbA1c 🡣FI No significant changes in PI and glucose levels

Olive leaf extract [133]	RCT, double-blind, placebo-controlled, crossover study, 12 weeks (OGTT, 75 g carbohydrates for postprandial measurement)	Olive leaf extract (51.1 mg oleuropein, 9.7 mg hydroxytyrosol)/day	Overweight middle-aged men, n=45	🡻AUC glucose 🡻AUC insulin 🡹insulin sensitivity (Matsuda index) 🡹disposition index 🡹pancreatic β-cell secretory capacity
Olive leaf extract [171]	RCT, double-blind, placebo-controlled, crossover study, 6 weeks	500 mg olive leaf extract, 100 mg green coffee bean extract, 150 mg beet powder (per capsule)/day	Adults with untreated high normal or borderline elevated BP, n=37	No significant change in FBG, insulin and HOMA-IR
Olive leaf extract [172]	RCT, double-blind, placebo-controlled, crossover study, 6 weeks	20ml (136 mg oleuropein, 6 mg hydroxytyrosol)/day (liquid)	Pre-hypertensive males, n=60	No significant change in FBG, insulin, HOMA-IR, QUICKI and fructosamine
Olive leaf extract [173]	8 acute studies conducted: 1 and 2: RCT, double-blind, placebo-controlled, crossover study Study 3: RCT, controlled, crossover study Studies 4-8: RCT, controlled, crossover study	1 and 2: 500 mg olive leaf extract in capsule (100 mg of oleuropein), or 1000 mg olive leaf extract (200 mg oleuropein) with 109 g of bread (50 g carbohydrates) Study 3: 100 g de-pitted olives (35 mg oleuropein) with 109 g bread (50 g carbohydrates) 4-7: 125 mg olive leaf extract (50 mg oleuropein) in water either with white or wholemeal bread, glucose or sucrose (50 g carbohydrates) Study 8: 0.4 g of OLE (160 mg oleuropein) with 25 g sucrose	Healthy participants: 1 and 2: n=24 Study 3: n=16 Studies 4-8: n=10	Consumption of olive leaf extract in capsules with white bread did not influence PG over 3 h Consumption of olives, or olive leaf extract in solution, with white or wholemeal bread also produced no significant changes in PG Higher doses of olive leaf extract with 25 g sucrose consumption 🡻PG peak and iAUC glucose
Olive leaf extract [174]	RCT, double-blind, placebo-controlled, crossover pilot study, 1 week (OGTT, 25 g sucrose solution)	150 mg oleuropein/day	Healthy women, n=11	No significant changes in postprandial Glc_{max}, time to reach Glc_{max}, and iAUC sucrose

Olive leaf extract [175]	RCT, parallel study, 12 weeks	330 mL of olive leaf tea 3 times/day	Prediabetes, n=57 (n=28 in olive leaf tea group, n=29 in low olive leaf tea group)	🡻FBG in higher olive leaf tea dose No significant change in HbA1c, FI, and HOMA-IR

AUC glucose: area under the curve of glucose; AUC insulin: area under the curve of insulin; BP: blood pressure; CVD: cardiovascular disease; FBG: fasting blood glucose; FI: fasting insulin; Glc_{max}: glucose maximum concentration; HbA1c: glycated haemoglobin A1c; HOMA-IR: homeostatic model assessment of insulin resistance; iAUC glucose: incremental area under the curve of glucose; iAUC sucrose: incremental area under the curve of sucrose; PG: postprandial glucose; PI: postprandial insulin; QUICKI: quantitative insulin sensitivity check index; RCT: randomised controlled trial; T2DM: type 2 diabetes mellitus; 2hPG: 2h postprandial glucose; 🡻means a decrease in value in the clinical outcome

2.6.1 New Zealand pine bark extract

Pine bark extract has stimulated much interest in its potential health benefits, and more recently in its hypoglycaemic properties in those with impaired glucose metabolism and diabetic complications such as microangiopathy and retinopathy [176, 177].

However, most of the research has been done on French maritime pine bark, also known as Pycnogenol, which is produced from the outer bark of *Pinus pinaster* Ait. Subsp. *Atlantica* growing in the Southwest coastal region in France [178], standardised to contain approximately 70% of procyanidins, [179]. Clinical studies to date on pine bark extract were chronic trials spanning from two weeks to six months, with doses from 50 to 480 mg/day (**Table 2.4**). Studies focusing on T2DM indicated significant improvements in glycaemic control [148, 149, 152], whereas other studies with other chronic conditions showing mixed hypoglycaemic responses with pine bark extract consumption [145-147, 150, 151, 153-157].

The New Zealand pine bark extract (Enzogenol®), is produced from *Pinus radiata* trees grown in New Zealand by a water-based extraction [180, 181]. The dry powder contains greater than 80% proanthocyanidins, 1–2% taxifolin, other flavonoids and phenolic acids, and some carbohydrates [181]. The proanthocyanidin content in the New Zealand pine bark was also shown to be even higher than Pcynogenol® [179]. Only three *in vivo* studies (mouse model and two human studies) to date have been conducted specifically on the New Zealand pine bark obtained from *Pinus radiata* trees and its impact on glycaemia, CVD risk factors and inflammation [147, 182, 183]. In the mouse model fed with the New Zealand pine bark significant improvements in diabetes-related biomarkers with a reduction in HbA1c, insulin, and glucagon levels, and an elevation of hepatic AMP-activated protein kinase (AMPK) activity were observed [182]. In the pilot study conducted by Shand et al. (2003) with healthy older participants beneficial effects on a range of CVD risk factor endpoints including reductions in weight and blood pressure (BP) were shown [147]. A further study done by Young et al. (2005) on the impact of the New Zealand pine bark on endothelial function and inflammation concluded no significant changes to glucose levels in chronic smokers after 12 weeks, although there was significant reduction in oxidative stress [183]. However, in both studies vitamin C was added in the mixture together with pine bark (480 mg/day) as part of the intervention, hence study outcome benefits could not be attributed solely to pine bark [147, 183]. More clinical research in humans is warranted to see if pure extracts of the New Zealand pine bark have hypoglycaemic effects on glycaemia.

2.6.2 Grape seed extract

The grape seed extract is isolated from the seeds of white grapes, *Vitis vinifera*, in the Southwest of France [184]. Research has shown the health beneficial effects of grape seed extract on diabetes [185] and cardiovascular conditions [186]. Studies looking at grape by-products reported that the seeds particularly contained high amounts of phenolics, compared to stems or pomace (seed and skin) [187, 188]. Flavonoids such as procyanidins, also known as proanthocyanidins or condensed tannins, are the main bioactive components found in grape seed extract [184, 189]. Various reviews have elucidated proanthocyanidins as modulators of glucose metabolism [190-192].

Grape seed extract has been more extensively studied compared to the other extracts (**Table 2.4**). Studies comprised of both acute and chronic trials spanning four to 12 weeks, with doses from 150 to 1300 mg/day for chronic trials and 100 to 500 mg for acute trials. Some studies reported significant reductions in postprandial glucose (PG) in healthy individuals and those with metabolic syndrome [132, 164, 165], whilst other studies on healthy participants and those with T2DM or metabolic syndrome did not show similar effects [158-163, 166]. The hypoglycaemic potential of grape seed has yet to be explored in people with prediabetes.

2.6.3 Rooibos tea extract

Rooibos, also known as *Aspalathus linearis*, or redbush, contains a rich source of glycosylated polyphenols, which have been shown to have potential benefit in diabetic conditions from studies conducted *in vitro* [193, 194] (**Table 2.4**). Rooibos tea is a caffeine-free beverage containing low amounts of tannins but is polyphenol-rich, generally including C-glucosyl dihydrochalcones (aspalathin and nothofagin), phenylpropenoids (phenylpyruvic acid-2-*O*-glucoside), C-glycosyl-containing flavones (isoorientin and orientin) and flavonols (quercetin-3-*O*-robinobioside) [142, 193]. Rooibos tea that is not fermented (green) has been demonstrated to contain three times the total phenolic content than the fermented extract [193], of which is also used in the GLARE study (Chapter 5).

To date, only one human clinical study has been conducted to examine the acute effects of unfermented rooibos tea on postprandial blood glucose as the primary outcome. Chepulis et al. (2016) showed that the consumption of rooibos tea extract significantly reduced postprandial blood glucose by approximately 34% in healthy individuals [132]. This gives an indication that rooibos tea extract could potentially alleviate poor glycaemic control in prediabetes. Other clinical studies relating to comorbidities of diabetes looked at the

consumption of rooibos tea and reductions in angiotensin-converting enzyme activity [195], improvements in markers for blood lipid levels and oxidative stress [167], and also increased antioxidant capacity in healthy humans [196]. Clinical research is required to determine the glucose modulating effects of unfermented green rooibos tea extract for people with prediabetes.

2.6.4 Olive leaf extract

Olive leaf obtained from the olive tree (*Olea europaea L.*) has been consumed in the Mediterranean region as a form of traditional herbal tea [197]. Phenolic compounds from olive leaf have been known to be high in antioxidant activity [143, 187]. The most abundant bioactive components in olive leaf is the secoiridoid compound oleuropein, followed by hydroxytyrosol, apigenin-7-glucoside and luteolin-7-glucoside, and verbascoside [198-200] and the compositions change with maturation or processing [201]. Lately, olive leaf has been implicated in improving metabolic syndrome risk factors [136], ameliorating high blood pressure [172, 202], improving lipid metabolism [203], and appears to possess cardio-protective [204], anti-inflammatory effects [143], and hypoglycaemic benefits [136].

Chronic studies on olive leaf extract spanned from one to 14 weeks, with doses 60 to 1000 mg/day (**Table 2.4**). Acute studies used doses of between 125 and 1000 mg along with oral glucose tolerance test (OGTT). Various forms of olive leaf extract were tested, mainly in capsules [133, 169-171, 173] but also in the form of liquid [172] and tea [175], as well as with real olive fruit [173]. Some trials with diabetic, hypertensive and overweight individuals demonstrated significant improvements in either fasting or postprandial glycaemic responses such as insulin sensitivity, β-cell function, 2hPG, HbA1c, FI, and FBG [133, 168, 170, 175]. However, other studies conducted in individuals with metabolic syndrome, in particular hypertensive individuals, did not demonstrate significant improvement in glucose response and insulin sensitivity with olive leaf extract, likely because their primary endpoint was blood pressure [169, 171, 172].

There have been two studies examining effect of olive leaf extract in a prediabetes cohort and a group that may have included participants with T2DM and prediabetes [168, 175]. Araki and colleagues (2019) conducted a 12-week study where participants were given olive leaf extract, but in the form of a tea beverage [175]. They showed that the higher dose of olive leaf tea significantly reduced FBG but not HbA1c, FI and HOMA-IR [175]. This study was reported since the GLARE study was completed. An earlier study by Komaki and co-workers (2003) investigated the anti-α-amylase components of olive leaf extract [168].

They also conducted a small, acute study in 14 healthy participants who consumed 1000 mg of olive leaves with 300 g of cooked rice to examine potential α-amylase inhibition in humans [168]. In a subgroup analysis the study outcome showed that participants in the borderline diabetic group (n=7; FBG of 6.1-7.8 mmol/L) demonstrated a significant reduction in PG [168]. However, the greater range used to define the borderline diabetic group means that whilst some participants could have had prediabetes others were likely to have T2DM.

2.7 Hypoglycaemic mechanisms of action of plant extracts

In the last decade, focus has begun to shift towards more complex biological modes of action of plant extract polyphenols in the body, such as their ability to improve glucose metabolism via modulating different metabolic pathways such as glycolysis, Krebs cycle, gluconeogenesis and carbohydrate metabolism [7, 86, 128, 139, 190, 205-211]. Different mechanisms of action exhibited by plant extracts to improve postprandial glycaemia include inhibiting hepatic gluconeogenesis and suppressing glucagon release, enhancing incretin effect and insulin response, delaying carbohydrate digestion by inhibiting α-amylase and α-glucosidase, inhibition of sodium-dependent glucose co-transporter-1 (SGLT1) and sodium-independent glucose transporter-2 (GLUT2) to prevent or delay glucose absorption and uptake [7, 208, 212, 213].

The hypoglycaemic effect of plant polyphenols is derived from their unique structural properties such as the number and position of hydrogen moieties (e.g. OH) and double bonds that determine their bioavailability and subsequent interaction with membrane-bound brush border enzymes, apically located transporters and receptors involved in glucose metabolic pathways [214-219]. Their hypoglycaemic effects often depend on the cultivars and variety, environmental factors such as season, climate, topography and soil type, method of extraction and treatment to retain or enhance the potency of the polyphenols [140, 220-223].

Figure 2.2 summarises how dietary sources of polyphenols from plants can potentially impact multiple organ levels via various mechanisms of glucose metabolism in a holistic manner to improve glycaemic control. This is an advantage over anti-diabetic drug therapy, although often well-characterised, but nonetheless exhibits solitary actions that disrupt metabolic equilibrium within the body [2]. Nutritional studies have been designed to elucidate the underlying mechanisms of action of natural plant extracts likened to pharmacological functionalities of anti-diabetic drugs, but with minimal to no adverse effects [7, 86, 128, 139, 190, 205-211]. Taking a multimodal approach such as with plant extracts is

highly attractive for the treatment of hyperglycaemia and T2DM due to the heterogeneous nature of T2DM involving multifaceted aetiologies [224].

Table 2.5 shows the mechanistic studies that have been conducted to date for the specific plant extracts (New Zealand pine bark, grape seed, rooibos tea, and olive leaf) that are examined in this PhD work. Although there may be multiple underlying mechanisms of action involved in the potential hypoglycaemic impact of plant extracts, a feasible mechanistic study on the enzyme inhibition of digestive enzymes: α-amylase and the dipeptidyl peptidase-4 (DPP4) enzyme, has been identified to be relevant to this PhD work.

Alpha-amylase is an important brush-border digestive enzyme located in the intestine and is involved in the breaking down of carbohydrates or starch into maltose and maltotriose. These sugars are further broken down by α-glucosidase to release glucose for absorption [213]. The inhibition of α-amylase may play a critical role in controlling the amount of carbohydrate digestion and reducing the glucose available for absorption and uptake, potentially leading to improved glycaemia [213]. The inhibition study employs a simple *in vitro* enzymatic assay methodology and for this advantage it has been widely used in research to screen for potential inhibitors in plant extracts and their phenolic components [225-229]. Using this method, many plant extracts have been identified to exhibit inhibitory action against α-amylase [225-229].

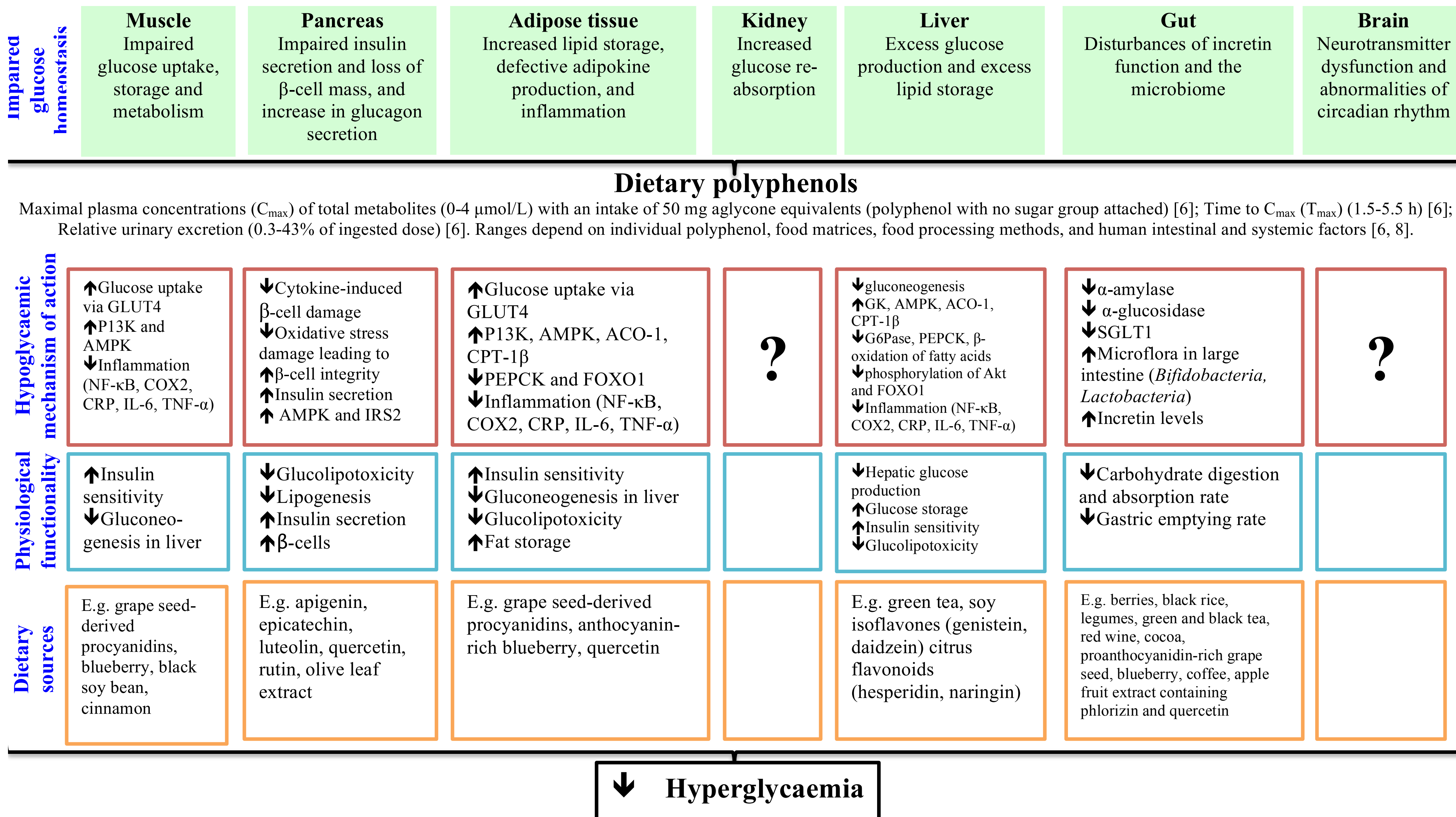
Several *in vitro* and *in vivo* studies have investigated the inhibitory effects of extracts of pine bark [230-232], grape seed [216, 233-236], rooibos tea [237, 238], and olive leaf [168, 170, 173, 174, 236, 239-242] on digestive enzymes such as α-amylase although comparative results are inconclusive due to different methodologies, types and concentrations of products tested. Therefore, this mechanistic action of inhibition on α-amylase will be explored in the New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts. The understanding of the potential inhibition on α-amylase will help inform future chronic trials looking at the impact of these plant extracts on postprandial glycaemia in the prediabetes cohort.

The glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are responsible for the incretin effect that helps reestablish PG homeostasis. However, decreased incretin concentrations have been observed in prediabetes and T2DM [243-252]. Furthermore, the incretins are degraded by DPP4 enzyme rendering them functionally inactive to participate in glycaemic control [253-256]. In addition, research has advocated that it is pivotal to restore β-cell function in prediabetes and not just treating its symptoms in order to sustain glycaemic control and delay T2DM development [257-260]. An effective

way is to enhance the incretin effect to amplify insulin response without overworking the β-cell [260]. Therefore, interventions aimed at restoring the incretin effect may be useful.

Hence, the investigation of the potential inhibition of DPP4 enzyme by New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts will help elucidate if the incretin levels can be preserved and amplify the incretin effect. More details regarding the mechanistic work can be found in Chapter 6 of the thesis.

Multifaceted channels of dietary polyphenols to improve glycaemic control in prediabetes and diabetes [1-4]



ACO-1: acyl CoA oxidase-1; AMPK: 5' adenosine monophosphate-activated protein kinase; COX2: cyclooxygenase-2 protein; CPT-1β: carnitine palmitoyl transferase-1β; CRP: C-reactive protein; FOXO1: forkhead box protein O1; G6Pase: glucose-6-phosphatase; GK: glucokinase; GLUT4: glucose transporter 4; IL-6: interleukin-6; IRS2: insulin receptor substrate 2; MCP-1: monocyte chemoattractant protein-1; NF-κB: nuclear factor kappaB; PI3K: phosphoinositide 3-kinase; PEPCK: phosphoenolpyruvate carboxykinase; SGLT1: sodium-dependent glucose transporter; TNF-α: tumour necrosis factor α

Figure 2.2 Hypoglycaemic potential of dietary polyphenols

Table 2.5 *In vitro* and *in vivo* studies on the hypoglycaemic mechanistic actions of plant extracts and their phenolic components examined in the PhD study

Mechanistic actions	Pine bark extract		Grape seed extract		Rooibos tea extract		Olive leaf extract	
	In vitro	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*
Insulin secretagogue or reduction?	[261]		[188, 262, 263]	[263-265]	[266]	[267, 268]	[269-272]	[273-275]
Increase in amylin or inhibition of amyloid fibril aggregation?				[276]	[277]		[271, 278-285]	
Glucokinase activator?		[182]		[286]	[287]			
Increase in adiponectin levels?		[288]		[134, 158, 289-291]	[292]	[293]		[275, 294]
Insulin sensitisation via peroxisome proliferator-activated receptors?	[295]	[182, 296]			[287, 292, 297, 298]			[275, 294]
Inhibition of hepatic gluconeogenesis?		[182]		[289, 290, 299, 300]		[293]		
Inhibition of glucagon release?		[182]						
Inhibition of glycogen synthase kinase-3?		[182]		[264, 290]				
Inhibition of digestive enzymes?	[230, 231]	[231]	[216, 233-236, 301, 302]	[301]	[237, 238, 303]		[168, 170, 173, 174, 236, 239-242, 304]	[173, 305]
Inhibition of lipase activity?	[288]		[216, 236, 306]	[307]			[236]	
Inhibition or enhanced translocation of glucose transporters to promote glucose uptake?	[308, 309]		[144, 265, 310]	[290]	[268, 287, 293, 297, 311-314]	[268]	[173, 174, 315-318]	[275, 317]
Increase in incretin levels?			[319, 320]	[321-324]			[325]	[325]
Inhibition of dipeptidyl peptidase-4 enzyme?			[322, 326]	[321, 322, 326]				
Inflammation/oxidative stress reduction?	[135, 327-330]	[148, 151, 153-157, 331-338]	[289, 339, 340]	[134, 158-164, 166, 291, 341-351]	[277, 293, 312, 352-360]	[138, 167, 196, 293, 352, 354, 359, 361-367]	[270, 318, 368]	[133, 172, 242, 275, 369-379]
Targeting signalling pathways of glucose metabolism?	[308]	[182, 288]	[264, 265, 310, 380, 381]	[264, 265, 276, 289, 290, 382-385]	[268, 277, 292, 293, 297, 312-314, 358, 386, 387]	[196, 268, 293, 366, 388, 389]	[173, 174, 269, 315, 317, 318, 390]	[294]

The areas highlighted in grey are areas of potential research in order to elucidate the specific glucose-lowering mechanisms of action of these plant extracts.

2.8 Conclusion

The number of individuals diagnosed with prediabetes and progressing to T2DM continues to rise. Alternative treatments to improve glycaemic control are needed as current pharmacological and lifestyle changes are not reducing the increasing prevalence. Antioxidant-rich New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts have been shown to be potential hypoglycaemic modulators and may be used as an adjunct to anti-diabetic drugs to improve glycaemic control in prediabetes. This review has identified the gaps in knowledge of these plant extracts and their potential hypoglycaemic effects in humans, with more research warranted in the prediabetes cohort. Additionally, the mechanisms of action underlying the desired hypoglycaemic effects such as the investigation of potential enzyme inhibition of digestive enzyme α-amylase and DPP4 enzyme have been identified and will be carried out as part of this PhD study.

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Chapter 3

Impact of phenolic-rich plant extracts on prediabetes and its subgroups. A narrative review of human clinical trials on prediabetes

This chapter discusses randomised controlled trials that have investigated the effects of phenolic-rich plant extracts on glycaemic control in people with prediabetes. This report has been presented in manuscript format and prepared for submission to the *Critical Reviews in Food Science and Nutrition* Journal.

Abstract

Phenolic-rich plant extracts have been demonstrated to improve glycaemic control in individuals with prediabetes. However, there is increasing evidence that people with prediabetes are not a homogeneous group but exhibit different glycaemic profiles leading to the existence of prediabetes subgroups. Prediabetes subgroups have been identified as: isolated impaired fasting glucose (IFG), isolated impaired glucose tolerance (IGT), and combined impaired fasting glucose and glucose intolerance (IFG/IGT). The present review investigates human clinical trials examining the hypoglycaemic potential of phenolic-rich plant extracts in prediabetes and prediabetes subgroups. *Artemisia princes* Pampanini, soy (Glycine max (L.) Merrill) leaf and *Citrus junos* Tanaka peel have been demonstrated to improve fasting glycemia and thus may be more useful for individuals with IFG with increasing hepatic insulin resistance. In contrast, white mulberry (*Morus alba* Linn.) leaf, persimmon (*Diospyros kaki*) leaf and *Acacia. Mearnsii* bark were shown to improve postprandial glycemia and hence may be preferably beneficial for individuals with IGT with increasing muscle insulin resistance. *Elaeis guineensis* leaf was observed to improve both fasting and postprandial glycaemic measures depending on the dose. Current evidence remains scarce regarding the impact of the plant extracts on glycaemic control in prediabetes subgroups and therefore warrants further study.

Keywords: functional food; polyphenol; impaired glycaemic control; impaired glucose tolerance; impaired fasting glucose

3.1 Introduction

Globally, diabetes rates have been increasing at an alarming rate. In 2019 it was estimated that 463 million (ages 20-79 years) (9.3%) people were living with diabetes worldwide, an increase of 62% from 2009 with the number expected to increase to 700 million (10.9%) by 2045 [1]. According to the International Diabetes Federation (IDF) the current annual global health expenditure on diabetes is estimated to be USD 760 billion and is projected to reach USD 845 billion by 2045 [1].

Much of the health costs come from the complications that are associated with diabetes, which can affect the eyes, kidneys and nervous system [2, 3], and heightens the risk of cardiovascular morbidity and mortality [4].

Although the rates of Type 1 diabetes mellitus have also been increasing the main driver of the increased rates of diabetes, it is the increase in the rates of Type 2 diabetes mellitus (T2DM) that constitutes approximately 90% of diabetes worldwide [1]. This has largely occurred in parallel with the obesity epidemic. Given the burden is and will put on health systems it is therefore crucial to identify strategies that would prevent or slow the development of T2DM.

Prediabetes is an intermediate state of hyperglycaemia with blood glucose levels above normal but not high enough to be classified as T2DM [5]. Prediabetes is a high-risk state for developing T2DM [6] and has an annual conversion rate of 5-10% [5, 7]. Therefore, early detection of prediabetes in conjunction with effective interventions may reduce the risk of developing future T2DM [8, 9].

There is increasing awareness that individuals with prediabetes are not a homogeneous group [10, 11] and show different metabolic profiles reflecting varying degrees of insulin resistance and β-cell dysfunction as observed in people with T2DM [10]. Three subgroups of glucose intolerance have been identified of which are the impaired fasting glucose (IFG), isolated impaired glucose tolerance (IGT), and combined impaired fasting glucose and impaired glucose tolerance (IFG/IGT) [10, 12]. These subgroups have distinctly different glycaemic metabolic profiles [7, 13-21], and exhibit different postprandial glucose (PG) and postprandial insulin (PI) shapes after a carbohydrate load [17, 20-23].

According to the American Diabetes Association (ADA) guidelines, individuals with isolated IFG have elevated fasting blood glucose (FBG) of 100-125 mg/dL (5.6-6.9 mmol/L) while having a normal 2h postprandial glucose (2hPG) of <140 mg/dL (<7.8 mmol/L) [24]. Individuals with IFG tend to exhibit increased endogenous glucose production (EGP),

reduced hepatic insulin sensitivity, stationary β-cell dysfunction and/or chronic low β-cell mass, defective early phase insulin secretion while maintaining normal second phase insulin secretion with PG returning to normal after 2h, altered glucagon-like peptide-1 (GLP-1) secretion and inappropriately elevated glucagon secretion [12, 13, 20-23, 25-33]. They tend to also possess healthy or near healthy peripheral insulin sensitivity [20, 25, 27, 29].

Individuals with isolated IGT typically have normal FBG of <100 mg/dL (<5.6 mmol/L), but an abnormally elevated 2hPG of 140-199 mg/dL (7.8-11.0 mmol/L) [24]. Characteristics specific to IGT may include increased or normal EGP, reduced peripheral insulin sensitivity, near-normal hepatic insulin sensitivity, impaired early and late phase insulin secretion with a subsequent rise in PG that is unable to return to normal baseline after 2h, persistent and progressive loss of β-cell function, reduced secretion of gastric inhibitory polypeptide (GIP) and inappropriately elevated glucagon secretion [12, 13, 20-23, 25-30, 33-35].

Individuals with combined IFG/IGT fulfill both criteria of having elevated FBG of 100-125 mg/dL (5.6-6.9 mmol/L) and elevated 2hPG of 140-199 mg/dL (7.8-11.0 mmol/L) [24]. IFG/IGT takes the worse form of impaired glucose control with a reduced glucagon suppression, impaired hepatic and peripheral insulin sensitivity and progressive loss of β-cell function, with a sustained rise in PG that does not return to normal baseline after 2h [20, 21, 29, 36, 37].

Anti-diabetic pharmacological drugs have targeted various organs such as muscle, pancreas, liver and gut responsible for glucose metabolism with specific mechanisms of action to improve glycaemic control [38]. Drugs such as metformin belonging to the class of biguanide, sulfonylureas, and meglitinides are insulin secretagogues and insulin sensitisers that target liver insulin resistance and suppress endogenous glucose production that could potentially improve fasting glycaemic responses and hence can be utilised to treat IFG [20, 39, 40]. In contrast, drugs targeting peripheral or muscle insulin resistance to improve skeletal muscle insulin sensitivity such as peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists, as well as α-glucosidase inhibitors, GLP-1 agonists, DPP4 inhibitors and thiazolidinediones that are most efficacious when taken together with a meal could potentially improve postprandial glycaemic responses and hence may be better utilised by those with IGT [20].

Phenolic-rich plant extracts have increasingly been known for their hypoglycaemic effects [41-43], and have the potential to be used as an alternative to anti-diabetic medications but with few to no adverse effects such as abdominal discomfort or weight gain

[38, 44]. Similarly, plant extracts have been shown to possess different hypoglycaemic mechanisms of action to affect glucose regulation in the human body [43, 45-53].

The question is whether plant extracts could emulate how these pharmacological agents are being categorised for a more effective, targeted clinical outcome for individuals in each prediabetes subgroup. A deeper understanding of the impact of plant extract interventions on prediabetes subgroups could enable the development of more targeted treatment strategies, with greater potential for slowing or stopping the development of T2DM.

In order to obtain results that elucidate the impact of interventions on individuals with varying degrees of dysglycaemia [10, 54, 55], stratification based on the glycaemic profile of the cohort is important. This will enable more specific identification of interventions appropriate for those having worsening glycaemic profiles [56-59].

The present review therefore aims to 1) investigate human clinical trials that have been conducted to examine the impact of plant extracts on glycaemic responses in individuals with prediabetes, and 2) examine the effectiveness of each plant extract intervention in the prediabetes subgroups.

3.2 Human clinical trials examining effect of plant extracts on glycaemic responses in the prediabetes cohort

Acute and chronic human clinical trials on plant extracts and involving participants with prediabetes were considered based on the ADA definition for prediabetes: IFG (FBG of 100-125 mg/dL and/or 2hPG <140 mg/dL), IGT (2hPG of 140-199 mg/dL and/or FBG <100 mg/dL) and IFG/IGT (FBG of 100-125 mg/dL and 2hPG of 140-199 mg/dL) [24]. Studies that included at least two glycaemic measurement outcomes such as fasting glycaemic indices: FBG, fasting insulin (FI), fasting C-peptide (FCP), and homeostasis model assessment-insulin resistance (HOMA-IR), and postprandial glycaemic indices: PG, PI, postprandial C-peptide (PCP), and glycated hemoglobin A1c (HbA1c) were included. Only those published in English were considered. Studies that have incorporated other administered therapies such as lifestyle modifications (e.g. physical activity) or concomitant glucose-lowering medications, or that involved fruit-based extracts, spices, and traditional Chinese medicine were beyond the scope of this review and therefore excluded.

Ten RCT studies including one randomized, uncontrolled, parallel study covering eight different plant extracts and their impact on glycaemic responses in prediabetes were

identified for this review (**Table 3.1**). Two of the identified studies were acute studies and the rest chronic studies of intervention duration ranging from 4 weeks to 12 weeks. Plant extracts examined were *Artemisia princes* Pampanini (Sajabalssuk) [60, 61], *Elaeis guineensis* leaf [62], *Ficus deltoidea* leaf [62], soy (Glycine max (L.) Merrill) leaf [63, 64], white mulberry (*Morus alba* Linn.) leaf [65-67], persimmon (*Diospyros kaki*) leaf [68], *Citrus junos* Tanaka peel [69], and *Acacia Mearnsii* bark [70]. Nine trials involved participants with IFG. One trial recruited participants with IGT. Three trials recruited participants with combined IFG/IGT. All plant extracts examined were able to elicit certain improvement in either fasting glycaemic measures such as FBG, FI, FCP and HOMA-IR, or postprandial glycaemic responses such as PG, PI, PCP, as well as HbA1c in participants with prediabetes, except *Ficus deltoidea* leaf.

Table 3.1 Human clinical trials involving plant extracts and their hypoglycaemic impact in participants with prediabetes

Plant extract	Study design	Participants (Type and total analysed sample size)	Treatment dose	Duration	Glycaemic measurements	Findings	Reference
Sajabalssuk (*Artemisia princeps* Pampanini)	RCT, parallel study	Prediabetes IFG (n=99)	Placebo, positive control or 3000 mg/day	9 weeks	FBG, FI, HOMA-IR, HbA1c, lipid profile (TG, TC, HDL, non-HDL, HTR, AI and PL), SBP, DBP, BMI, WHR, BFP, ALT and AST	Significant reduction in FBG and HbA1c compared to positive control, placebo and baseline. Significant reduction in HOMA-IR compared to placebo but not to positive control or baseline. No significant change in FI compared to positive control, placebo and baseline. Significant increase in HDL and decrease in non-HDL compared to positive control, placebo and baseline. Significant reduction in TC compared to positive control and baseline but not placebo. No significant changes in TG, HTR, AI, PL, SBP and DBP compared to positive control, placebo and baseline. No significant changes in BMI, WHR, BFP, ALT and AST compared to positive control, placebo and baseline.	[60]
Sajabalssuk (*Artemisia princeps* Pampanini)	RCT, parallel study	Prediabetes IFG and borderline diabetic (n=80)	Placebo or positive control 2000 mg/day 4000 mg/day	8 weeks	FBG, FI, FCP, HOMA-IR, glucagon, HbA1c, FFA, ALT, AST, SBP and DBP	Significant reduction in FBG and HbA1c with both doses compared to baseline. No significant changes in FI, FCP, HOMA-IR, glucagon and DBP with both doses compared to baseline. Significant reduction in FFA and SBP with higher dose (4000 mg/day) compared to baseline. Significant reduction in AST with both doses compared to baseline and a significant reduction in AST with lower dose (2000 mg/day) compared to positive control, but no significant change in ALT with both doses compared to baseline.	[61]

Elaeis guineensis leaf	Randomised, parallel study	Prediabetes IFG (n=9)	500 mg/day	8 weeks	FBG, FI, insulin sensitivity (%), HOMA-IR, PG AUC, PI AUC, BW and WC	Significant reduction in FBG, FI, insulin sensitivity (%) and WC compared to baseline, but no significant changes in HOMA-IR, PG AUC, PI AUC and BW compared to baseline.	[62]
		Prediabetes IFG (n=10)	1000 mg/day			Significant reduction in PG AUC, PI AUC and WC compared to baseline but no significant changes to FBG, FI, HOMA-IR, insulin sensitivity (%) and BW compared to baseline.	
Ficus deltoidea leaf	Randomised, parallel study	Prediabetes IFG (n=9)	1000 mg/day	8 weeks	FBG, FI, insulin sensitivity (%), HOMA-IR, PG AUC, PI AUC, BW and WC	No significant changes observed.	[62]
Soy (Glycine max (L.) Merrill) leaf	RCT, parallel study	Overweight and prediabetes IFG (n=30)	Placebo or 2000 mg/day	12 weeks	FBG, FI, HOMA-IR, HbA1c, BW, BMI, WC, WHR, BFP, lipid profile (TG, TC, HDL, LDL, HTR, and AI), ALT, AST, SBP and DBP	Significant reduction in FBG, HOMA-IR, HbA1c, WC, BFP, TG, AI, ALT and AST compared to placebo but not when compared to baseline. Significant increase in HDL and HTR compared to placebo but not when compared to baseline. No significant changes in FI, BW, BMI, WHR, TC, LDL, SBP and DBP compared to placebo and baseline.	[63]
Pterocarpan-high Soy (Glycine max (L.) Merrill) leaf	RCT, parallel study	Overweight and obese, with borderline metabolic syndrome and prediabetes IFG (n=44)	Placebo or 2000 mg/day	12 weeks	FBG, FI, HOMA-IR, HbA1c, BW, BMI, BFP, WHR, lipid profile (TG, FFA, TC, HDL, non-HDL, LDL, and AI), SBP, DBP, PAI-1, TNF-α, IL-6, MCP-1, adiponectin, and leptin, AST and ALT	Significant reduction in HOMA-IR and HbA1c compared to placebo and baseline. Significant reduction in FBG, FI, TC and SBP compared to baseline but not when compared to placebo. No significant changes to BW, BMI, BFP, WHR, DBP, AST and ALT compared to placebo and baseline. No significant changes to lipid profile except significant reductions in FFA and non-HDL compared to placebo and baseline. No significant changes to plasma adipokine and cytokine levels except significant reductions in PAI-1 and TNF-α compared to placebo and baseline, and significant reduction in IL-6 compared to baseline.	[64]

White mulberry (*Morus alba* Linn.) leaf and white kidney bean extract	RCT, parallel study	Prediabetes, IFG (n=65)	1500 mg (500 mg mulberry extract with 10% DNJ, 1000 mg white kidney bean extract)	Acute	PG iAUC, PI iAUC, PCP iAUC	Significant reduction in PG iAUC, PI iAUC and PCP iAUC compared to control group in the acute study.	[71]
			4500 mg/day (1500 mg/meal)	4 weeks		No significant changes to PG iAUC, PI iAUC, PCP iAUC HOMA-IR, HbA1c, and GSP compared to control group in the chronic study	
White mulberry (*Morus alba* Linn.) leaf and onion extract	RCT, parallel study	Prediabetes IFG (n=46)	Placebo or cooked rice coated with extract (8.8 mg DNJ) in experimental (prediabetes) and normal (healthy) groups	Acute	PG and PG AUC	Significant reduction in PG and PG AUC compared to placebo.	[65]
White mulberry (*Morus alba* Linn.) leaf	RCT, parallel study	Prediabetes IFG (n=65)	Placebo or extract with 6 mg DNJ	12 weeks	FBG, FI, GA, 1,5AG, HbA1c	Significant reduction in HbA1c from week 4 and GA from week 8 compared to baseline, but not when compared to placebo. No significant changes in FBG and FI compared to baseline and placebo. Significant increase in 1,5 AG from week 4, 8 and 12 compared to baseline, and overall significant increase compared to placebo.	[66]

White mulberry (*Morus alba* Linn.) leaf	RCT, parallel study	Prediabetes IFG (n=38)	Placebo or 5000 mg/day (18 mg DNJ)	4 weeks	PG and PG iAUC, PI and PI iAUC, PCP and PCP iAUC, ALT and AST	Significant reduction in PG and PI only at 30 min compared to placebo. Significant reduction in PCP at 30 and 60 min compared to placebo. No significant changes in PG iAUC, PCP iAUC, ALT and AST but only PI iAUC was significantly lower than placebo.	[67]
Persimmon (*Diospyros kaki*) leaf	RCT, parallel study	Prediabetes IGT (n=68)	Placebo or 2000 mg/day	8 weeks	PG	Significant reduction in PG compared to placebo.	[68]
Citrus junos Tanaka peel	RCT, crossover study	Prediabetes IFG/IGT (n=35)	Placebo, or 4250 mg/day	8 weeks	FBG, FI, FCP, PG, HOMA-IR	Significant reduction in FBG, FI and HOMA-IR compared to placebo but not when compared to baseline. No significant change in PG compared to placebo or baseline. No significant change in FCP when compared to placebo but significant reduction in FCP when compared to baseline.	[69]
Acacia. Mearnsii bark	RCT, parallel study	Prediabetes, IFG/IGT (n=34)	Placebo, or 1000 mg/day	8 weeks	FBG, FI, HOMA-IR, PG and PG AUC and PI and PI AUC and HbA1c	Significant reduction in PG at 90min and PI at 90 and 120 min compared to baseline. Significant reduction in PG at 120 min and PI at 90 min after 8 weeks compared to placebo. No significant changes in PG AUC and PI AUC compared to placebo but a significant reduction compared to baseline after 8 weeks. No significant changes in FBG, FI, HOMA-IR and HbA1c after 8 weeks compared to placebo and baseline.	[70]

White mulberry (*Morus alba* Linn.) leaf	RCT, crossover study	Prediabetes IFG/IGT (n=10)	Placebo	Acute	PG and PI	Not applicable.	[66]
			Extract with 3 mg DNJ			No significant change in PG compared to placebo but a significant reduction in PI at 30min compared to placebo.	
			Extract with 6 mg DNJ			Significant reduction in PG at 30min and significant reduction in PI at 30min compared to placebo.	
			Extract with 9 mg DNJ			Significant reduction in PG at 30min and significant reduction in PI at 30min compared to placebo.	

ALT: alanine aminotransferase; AI: atherogenic index; AST: aspartate aminotransferase; BFP: body fat percentage; BMI: body mass index; BW: body weight; DBP: diastolic blood pressure; DNJ: 1-deoxynojirimycin; FBG: fasting blood glucose; FCP: fasting C-peptide; FFA: free fatty acid; FI: fasting insulin; GA: glycated albumin; GSP: glycated serum protein; HbA1c: glycated hemoglobin A1c; HDL: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment-insulin resistance; HTR: high-density lipoprotein cholesterol (HDL) to total cholesterol (TC) ratio; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; IFG/IGT: combined impaired fasting glucose and impaired glucose tolerance; IL-6: interleukin-6; LDL: low-density lipoprotein cholesterol; MCP-1: monocyte chemotactic protein-1; PAI-1: plasminogen activator inhibitor-1; PCP: postprandial C-peptide; PCP iAUC: incremental area under the curve of postprandial C-peptide; PG: postprandial glucose; PG AUC: area under the curve of postprandial glucose; PG iAUC: incremental area under the curve of postprandial glucose; PI: postprandial insulin; PI AUC: area under the curve of postprandial insulin; PI iAUC: incremental area under the curve of postprandial insulin; PL: phospholipid; SBP: systolic blood pressure; TC: total cholesterol; TG: triglyceride; TNF-α: tumor necrosis factor-α; WC: waist circumference; WHR: waist-hip ratio; 1,5AG: 1,5-anhydroglucitol.

3.3 Effectiveness of plant extracts on glycaemic responses in the prediabetes subgroups

With the prediabetes cohort from these trials being classified into their respective subgroups, **Table 3.2** summarises the significant changes in glycaemic clinical outcomes of the interventions with the plant extracts based on each subgroup.

3.3.1 Hypoglycaemic effects of plant extracts on impaired fasting glucose (IFG)

Artemisia princeps Pampanini (*A. princeps*) belongs to one of the 500 plants under the genus *Artemisia*, and is commonly found in China, Korea and Japan [72, 73], and has been used to treat diabetes [74, 75]. High concentrations of antioxidants and flavonoids such as eupatilin and jaceosidin have likely contributed to its anti-diabetic effects [72, 73, 76, 77]. The Korean A. *princeps* or Sajabalssuk extract (3000 mg/day) has also been examined in a prediabetes cohort for its glucose-lowering effects [60]. There were significant reductions in FBG (-16.5 ± 2.8%, $p<0.05$) compared to placebo and positive control after nine weeks of intervention, thus restoring normal FBG levels. Sajabalssuk extract also significantly decreased HbA1c (-7.8 ± 3.4%, $p<0.05$) and insulin resistance (HOMA-IR) (-14.7 ± 20.4%, $p<0.05$) with improvement in high-density lipoprotein (HDL) cholesterol level ($p<0.05$) compared to control (**Table 3.2**) [60]. An earlier study conducted by the same group also showed significant reductions in FBG and HbA1c at both doses (2000 and 4000 mg/day) in participants with IFG and borderline T2DM (FBG 123.3 ± 5.7 – 125.8 ± 6.1 mg/dL) compared to participant baseline after eight weeks of intervention. High dose (4000 mg/day) of the extract was also able to significantly decrease plasma free fatty acid (FFA) levels ($p<0.05$) compared to participant baseline [61]. These chronic studies have demonstrated that sajabalssuk extract was able to improve fasting glycaemic responses in IFG participants.

Elaeis guineensis (*E. guineensis*) leaf comes from oil palm and is commonly found in Malaysia, Thailand, Indonesia, Africa and South America [62, 78, 79]. It has been known to contain high levels of antioxidant activity rich in phenolic compounds such as catechin, apigenin and luteolin [80, 81], and *in vitro* and animal studies have elucidated *E. guineensis* to be beneficial for metabolic syndrome and T2DM by promoting vascular relaxation and reducing inflammation and lipid oxidation [78, 80, 82-84]. Kalman and group investigated the hypoglycaemic effects of *E. guineensis* leaf extract at two doses (500 and 1000 mg) in participants with IFG for eight weeks [62]. *E. guineensis* leaf extract at a lower dose (500 mg) was able to significantly improve FBG ($p=0.02$), fasting insulin (FI) ($p=0.04$), and

HOMA-IR (*p*=0.03) compared to participant baseline levels (**Table 3.2**). In contrast, the higher dose (1000 mg) was only able to significantly improve PG and PI responses (*p*=0.046 and *p*=0.006, respectively) compared to participant baseline levels. Having no placebo group was a limitation of the study. Due to the paucity of clinical data regarding *E. guineensis* leaf, more research is required to investigate the glucose-lowering potential of *E. guineensis* leaf in people with prediabetes.

Ficus deltoidea (*F.deltoidea*) belongs to the Moraceae plant family and is native to the Malayan Archipelago [85]. It is high in phenolic content such as flavan-3-ol monomers, catechin and afzelechin and antioxidant activity [86, 87]. In the past decade *in vitro* and animal studies have shown *F.deltoidea* as a potential anti-diabetic treatment owing to its glucose-lowering effects and its ability to stimulate insulinotropic activity and glucose uptake [85, 88-93]. There was only one 8-week prospective, randomized, double-blind, parallel study conducted investigating the impact of a single dose of *F.deltoidea* leaf extract (1000 mg) on individuals with IFG [62]. No significant changes in glucose and insulin responses were observed (**Table 3.2**).

Soy (Glycine max (L.) Merrill) leaf is common in Korea and Japan [94-96]. Soy leaf is rich in polyphenols such as kaempferol glycosides, coumestrol and pterocarpan [95-98], which have been shown to contain anti-diabetic properties [95, 98, 99]. Choi and colleagues (2014) showed that consuming soy leaf extract (2000 mg/day) for 12 weeks led to significant reductions in baseline-adjusted FBG, HOMA-IR, HbA1c, and lipid profile in overweight participants with IFG compared to placebo ($p<0.05$) [63] (**Table 3.2**). This finding was with agreement with another RCT investigating the impact of pterocarpan-high soy leaf extract (2000 mg/day) for 12 weeks on glucose tolerance in overweight and obese IFG participants with borderline metabolic syndrome [64]. Significant reductions in HbA1c, HOMA-IR, FFA and non-HDL cholesterol were observed in the intervention compared to control group [64]. FBG and FI were also reduced after intervention compared to participant baseline [64]. The clinical outcomes suggest that that the intervention with soy leaf extract could potentially benefit those with IFG as seen in the improvements in fasting glycaemic indices (FBG and HOMA-IR), with the addition of improved long-term glycaemic measurement, HbA1c and improved lipid profile.

White mulberry (*Morus alba* Linn.) leaf comes from the mulberry tree belonging to the family Moraceae and is native to Korea, Japan and China but also widely cultivated in other parts in Europe [100]. Mulberry leaf has been extensively studied and reviews have been written regarding its hypoglycaemic effects [100-102]. A variety of polyphenols such as

quercetin, chlorogenic acid, and rutin, and nitrogen-containing glucose analog 1-deoxynojirimycin (DNJ) contained in mulberry leaf contribute to the hypoglycaemic effects observed [103-108]. DNJ has been shown as a strong α-glucosidase inhibitor due to its size and structural similarity to glucose [109, 110] and has been used to standardise mulberry leaf extracts, with other phenolic components in the leaf contributing to its combined inhibitory action [65, 103, 109, 111-114]. Considerable human studies have further elucidated the hypoglycaemic effects of mulberry leaf extract in healthy participants, with fewer studies on prediabetes and T2DM [65-67, 113, 115-123]. Liu and colleagues (2020) investigated the hypoglycaemic effects of an extract mixture of mulberry leaf and white kidney bean in participants with IFG [71]. A significant reduction in glycaemic responses of incremental area under the curve (iAUC) such as PG iAUC, PI iAUC and PCP iAUC was observed compared to control in the acute trial. In contrast, the same study did not observe similar improvements in a 4-week chronic trial [71]. Hwang and co-workers (2016) investigated the impact of 50% ethanolic extract of mulberry leaf (20% in mixture) with onion extract coated on 75 g cooked rice (11.77 ± 1.67 mg DNJ/ 100 g rice) and observed an improvement in PG ($p<0.05$) and postprandial glucose area under the curve (PG AUC) ($p<0.001$) after an oral glucose tolerance test (OGTT) (75g cooked rice) in participants with IFG compared to placebo group [65] (**Table 3.2**). Asai and co-workers (2011) observed a significant increase in serum 1,5-anhydroglucitol (1,5-AG) concentration, a sensitive marker of postprandial hyperglycaemic spikes, in participants with IFG after consuming mulberry leaf (6 mg DNJ) for 12 weeks ($p<0.001$) in comparison to control [66]. However no significant changes were found in FBG, FI, HbA1c, and glycated albumin (GA) concentrations compared to placebo, but HbA1c was significantly reduced from 4-week onwards within the intervention group compared to participant baseline (6.0 ± 0.4% vs 5.9 ± 0.3%, $p<0.05$) (**Table 3.2**). Kim and colleagues (2014) investigated the impact of 4-week mulberry leaf extract (5000 mg/day, 0.36% or 18 mg DNJ) in IFG participants and demonstrated significant reductions in PG, PI and postprandial C-peptide (PCP) especially at 30 min post-load compared to placebo [67] (**Table 3.2**). However, no significant changes were found in FBG, FI and HbA1c compared to placebo [67]. Studies on mulberry leaf extract on healthy participants and individuals with prediabetes or T2DM have consistently shown non-significant changes in FBG and FI [66, 67, 115, 121-123]. This may suggest that mulberry leaf extract, which is functionally similar to acarbose, may be more beneficial for individuals with IGT due to its inhibitory action on digestive enzyme (α-glucosidase) post-load. Future studies on mulberry leaf could ascertain the inhibition of α-glucosidase in participants with prediabetes using hydrogen tests and

starch ^{13}C breath test that have been conducted in healthy and T2DM participants to indicate carbohydrate indigestion [117-119, 123].

3.3.2 Hypoglycaemic effects of plant extracts on impaired glucose tolerance (IGT)

Persimmon (*Diospyros kaki*) leaf belongs to the family of *Ebenaceae* and has been traditionally used in Japan, South Korea and China as a folk medicine [124]. The persimmon leaf has shown to possess anti-oxidative properties mediated by its rich phenolic concentration [125-127]. Phenolic compounds such as triterpenoids isolated from persimmon leaf have been shown to exhibit anti-diabetic properties via inhibiting protein tyrosine phosphatase 1B (PTP1B) activity (>80% inhibition at 30 µg/mL) [128]. Vomifoliol, which is found in persimmon leaf, has been identified as a potent a α-glucosidase inhibitor and an enhancer of peripheral glucose utilisation [129]. Khan and colleagues (2017) demonstrated consuming 2000 mg of persimmon leaf extract for eight weeks in IGT participants led to significant PG reduction in the intervention group compared to control (p=0.029) [68] (**Table 3.2**). Within the same study, samples of saliva, urine, and serum collected from a subgroup of five participants with combined IFG/IGT were analysed for potential protein markers of persimmon leaf treatment. Outcomes showed Tamm-Horsfall protein, uromodulin, SPARC-like protein 1 precursor (SPARCL1) and Complement C7 were down-regulated while Ezrin was up-regulated, indicating ameliorating effects of persimmon leaf on glycaemia [68]. *In vitro* and animal studies on persimmon leaf have elucidated the mechanistic action of α-amylase and α-glucosidase inhibition [130-132], which might have led to the PG reduction observed in the IGT participants due to reduced carbohydrate digestion [68]. Another mechanism of action demonstrated by persimmon leaf might be the inhibition Na+/glucose co-transporter (SGLT1) as the final stage of glucose absorption, as demonstrated by significant reductions in PG in rats after glucose loading [132].

3.3.3 Hypoglycaemic effects of plant extracts on combined impaired fasting glucose and impaired glucose tolerance (IFG/IGT)

The *Citrus junos* Tanaka (*C. junos*) fruit, also known as yuja or yuzu, is a yellow citrus fruit easily obtainable in Japan, Korea and China, and contains a high concentration of phenolic content and vitamin C compared to the flesh [133-137]. The major phenolic compounds hesperidin and naringin [133], which have been known for improving glycaemia [138]. Hwang and co-workers (2015) determined the impact of *C. junos* peel extract on

glycaemic responses in participants with combined IFG/IGT [69]. After eight weeks of intervention (4250 mg/day), the intervention group showed significantly reduced FBG (p=0.049), FI (p=0.038), and HOMA-IR (p=0.019) compared to the placebo group [69]. C-peptide in the intervention group was also marginally reduced (p=0.057) but no significant improvement in PG compared to placebo [69] (**Table 3.2**). The study showed that *C. junos* peel could only improve fasting glycaemic indices in combined IFG/IGT participants [69]. The hypoglycaemic mechanism might be due to increased glucose uptake via increased insulin action in the peripheral tissues [136].

Acacia. Mearnsii (*A.mearnsis*) bark from the black wattle tree of the legume family has been gaining attention for its anti-diabetic effects [139, 140]. Its anti-diabetic potential may be attributed to the abundant antioxidants and proanthocyanidins, such as catechin-like flavan-3-ols, and in particular robinetinidol and fisetinidol present in the bark [139]. An 8-week consumption of *A. mearnsii* bark (1000 mg/day) led to significant reduction in PG at 120 min (p=0.013) and PI at 90 min (p=0.032) compared to placebo in participants with combined IFG/IGT [70]. There was also a significant reduction in glucose at 90 min (p=0.014), and a reduction in insulin concentration at 90 and 120 min (p=0.002 and p=0.004, respectively), with an overall reduction in PG AUC and postprandial insulin area under the curve (PI AUC) (p=0.018 and p=0.009, respectively) within the intervention group [70] (**Table 3.2**). However there was no change in fasting glycaemic measures such as FBG, FI, HOMA-IR, and HbA1c compared to placebo [70]. Mechanistic *in vitro* studies indicate that the postprandial hypoglycaemic effects of *A. mearnsii* bark could be due to inhibition of the digestive enzymes α-amylase and α-glucosidase [139-145]. This study suggests that *A. mearnsii* bark preferentially improved postprandial glycaemic responses instead of fasting glycaemic responses in IFG/IGT participants.

The mulberry leaf extract was also examined in participants with combined IFG/IGT by Asai and co-workers (2011) [66]. They found that the extract (3, 6 or 9 mg DNJ) significantly reduced acute PG responses in a dose-dependent manner compared to placebo (p=0.006) (**Table 3.2**). This indicates that mulberry leaf may preferentially improve postprandial glycaemic responses in individuals with IFG/IGT. As discussed earlier, mulberry leaf extract was shown to improve postprandial glycaemic responses in IFG participants as well.

Table 3.2 Changes in glycaemic clinical outcomes in participants with prediabetes classified by their subgroups. The significant outcomes are presented as comparison with control, then comparison within intervention group.

Plant extract	Dose	Fasting state				Postprandial state			HbA1c	Reference
		FBG	FI	FCP	HOMA-IR	PG/ PG AUC	PI/ PI AUC	PCP/PCP AUC		
Human clinical trials on impaired fasting glucose (IFG)										
Sajabalssuk (*Artemisia princeps* Pampanini)	3000 mg/day	↓, ↓	-, -	na	↓, -	na	na	na	↓, ↓	[60]
Sajabalssuk (*Artemisia princeps* Pampanini)	2000 mg/day	na, ↓	na, -	na, -	na, -	na	na	na	na, ↓	[61]
	4000 mg/day	na, ↓	na, -	na, -	na, -	na	na	na	na, ↓	
Elaeis guineensis leaf	500 mg/day	na, ↓	na, ↓	na	na, -	na, -	na, -	na	na	[62]
	1000 mg/day	na, -	na, -	na	na, -	na, ↓	na, ↓	na	na	
Ficus deltoidea leaf	1000 mg/day	na, -	na, -	na	na, -	na, -	na, -	na	na	[62]
Soy (Glycine max (L.) Merrill) leaf	2000 mg/day	↓, -	-. -	na	↓, -	na	na	na	↓, -	[63]
Pterocarpan-high Soy (Glycine max (L.) Merrill) leaf	2000 mg/day	-, ↓	-, ↓	na	↓, ↓	na	na	na	↓, ↓	[64]
White mulberry (*Morus alba* Linn.) leaf and white kidney bean extract	1500 mg	na	na	na	na	↓	↓	↓	na	[71]
	4500 mg/day	na, na	na, na	na, na	-, na	-, na	-, na	-, na	-, na	
White mulberry (*Morus alba* Linn.) leaf and onion extract	Cooked rice coated with extract (8.8 mg DNJ)	na	na	na	na	↓	na	na	na	[65]
White mulberry (*Morus alba* Linn.) leaf	Extract with 6 mg DNJ	-, -	-, -	na	na	na	na	na	-, ↓	[66]
White mulberry (*Morus alba* Linn.) leaf	5000 mg/day (18 mg DNJ)	-, na	-, na	-, na	na	↓, na	↓, na	↓, na	-, na	[67]

Human clinical trials on impaired glucose tolerance (IGT)										
Persimmon (*Diospyros kaki*) leaf	2000 mg/day	na	na	na	na	↓, na	na	na	na	[68]
Human clinical trials on combined impaired fasting glucose and impaired glucose tolerance (IFG/IGT)										
Citrus junos Tanaka peel	4250 mg/day	↓, -	↓, -	-, ↓	↓, -	-, -	na	na	na	[69]
Acacia. Mearnsii bark	1000 mg/day	-, -	-, -	na	-, -	↓, ↓	↓, ↓	na	-, -	[70]
White mulberry (*Morus alba* Linn.) leaf	Extract with 3 mg DNJ	-	-	na	na	-	↓	na	na	[66]
	Extract with 6 mg DNJ	-	-	na	na	↓	↓	na	na	
	Extract with 9 mg DNJ	-	-	na	na	↓	↓	na	na	

DNJ: 1-deoxynojirimycin; FBG: fasting blood glucose; FCP: fasting C-peptide; FI: fasting insulin; HbA1c: glycated hemoglobin A1c; HOMA-IR: homeostasis model assessment-insulin resistance; IFG: impaired fasting glucose; IFG/IGT: combined impaired fasting glucose and impaired glucose tolerance; IGT: impaired glucose tolerance; PCP: postprandial C-peptide; PCP AUC: area under the curve of postprandial C-peptide; PG: postprandial glucose; PG AUC: area under the curve of postprandial glucose; PI: postprandial insulin; PI AUC: area under the curve of postprandial insulin; RCT: Randomised controlled trial; ↓: a significant decrease in the measured value ($p<0.05$); -: no significant changes to measured value ($p>0.05$); na: not applicable.

3.4 Does each prediabetes subgroup benefit from different plant extracts?

Phenolic-rich plant extracts have been known to improve glucose regulation in individuals with prediabetes, however not all plant extracts may benefit both fasting and postprandial hyperglycaemia. **Table 3.2** shows how the different plant extracts can be more appropriately used for individuals with IFG, or IGT based on whether they could improve fasting or postprandial glycaemic responses, respectively. Plant extracts that were able to demonstrate improvements in fasting glycaemic indices such as FBG, FI and HOMA-IR were categorised as being useful for IFG. In contrast, plant extracts that demonstrated to improve postprandial glycaemic indices such as PG and PI were grouped as being helpful for IGT. This is likely due to the different phenolic structures of each plant extract that enables varying kinds of hypoglycaemic mechanisms of action [42, 48, 146, 147]. **Table 3.3** summarises how the discussed plant extracts can help individuals with IFG or IGT. Individuals with IFG/IGT are likely to benefit from both categories of glycaemic improvement.

Table 3.3 Hypoglycaemic effects of plant extracts on fasting and postprandial glycaemic measurements

Plant extracts with potential hypoglycaemic effects on fasting glycaemic measurements
Sajabalssuk (*Artemisia princeps* Pampanini)
Elaeis guineensis leaf (500 mg/day)
Soy (Glycine max (L.) Merrill) leaf
Citrus junos Tanaka peel
Plant extracts with potential hypoglycaemic effects on postprandial glycaemic measurements
White mulberry (*Morus alba* Linn.) leaf
Elaeis guineensis leaf (Higher dose, 1000 mg/day)
Persimmon (*Diospyros kaki*) leaf
Acacia. Mearnsii bark

3.5 Strengths and limitations

The merits of the present review were the inclusion of a range of human clinical trials investigating plant extracts with promising hypoglycaemic potential in individuals with prediabetes, and examination of the effectiveness of each plant extract intervention on the various prediabetes subgroups.

However, the review is not without limitations. The review has included single dose studies with some studies having only small sample sizes. More human studies are necessary to ascertain the hypoglycaemic impact of the plant extracts on people with prediabetes.

Most of the studies included did not clearly differentiate between the subgroups of prediabetes during recruitment. For example, nine studies that were included have only measured FBG to recruit participants with IFG, however these participants might also have IGT, but participant baseline PG during screening was not measured. This limitation highlights the importance of measuring both fasting and postprandial indices in future studies and for the purpose of classifying participants into the different prediabetes subgroups. Additionally, owing to the lower reproducibility with current measurements using FBG and 2hPG, caution should be exercised when classifying individuals into IFG or IGT based on a single test [11, 148]. However, the cost and practicality may need to be considered.

Furthermore, studies have not included both fasting and postprandial measurements. For example, the study on persimmon leaf extract only measured postprandial glycaemic response such as PG in IGT participants. On the other hand, studies on sajabalssuk and soybean leaf extract only measured fasting glycaemic responses without investigating postprandial glycaemic measures in IFG participants. Therefore the extensive impact of the plant extracts on both fasting and postprandial glycaemia could not be known.

Most of the studies included in this review did not take into account possible changes in β-cell function, insulin sensitivity and changes to lipid metabolism. This is important because the preservation or restoration of β-cell function is pivotal in slowing or halting the progression of prediabetes into T2DM [149-152]. In addition, dyslipidaemia often occurs in

prediabetes and may gradually impair insulin signaling and function and is therefore an important endpoint measurement in interventions [153].

3.6 Conclusion

This review has explored a new perspective in viewing how nutritional interventions could cater to the different classifications of prediabetes such as IFG, IGT and a combination of IFG/IGT. Among these studies, interventions with plant extracts have elucidated preferential improvements of certain glycaemic measurements. It follows that to obtain optimal glycaemic outcomes treatments should be made available that are specific for each of the prediabetes subgroup.

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Chapter 4
An acute, placebo-controlled, single-blind, crossover, dose-response, exploratory study to assess the effects of New Zealand pine bark extract (Enzogenol®) on glycaemic responses in healthy participants

This chapter presents the study outcomes investigating the effect of New Zealand pine bark extract on glycaemic control in healthy participants. This pine bark study has been presented in manuscript format and has been published in the *Nutrients* Journal.

Lim WXJ, Chepulis L, von Hurst P, Gammon CS, Page RA. An acute, placebo-controlled, single-blind, crossover, dose-response, exploratory study to assess the effects of New Zealand pine bark extract (Enzogenol®) on glycaemic responses in healthy participants. *Nutrients.* 2020;12(2).

Abstract

An acute, placebo-controlled, single-blind, crossover, dose-response, exploratory study was designed to investigate the hypoglycaemic effects of New Zealand pine bark extract (Enzogenol®). Twenty-five healthy participants categorised into having a monophasic or complex (biphasic or triphasic) glucose curve shape at the control visit consumed a placebo and Enzogenol® (50 and 400 mg) on three separate occasions before an oral glucose tolerance test (OGTT). In the monophasic group, 50 and 400 mg of Enzogenol® significantly reduced the mean glucose incremental area under the curve (iAUC) compared to control 241.3 ± 20.2 vs. 335.4 ± 34.0 mmol/L·min, p=0.034 and 249.3 ± 25.4 vs. 353.6 ± 31.5 mmol/L·min, p=0.012, respectively. The 400 mg dose further reduced the percentage increment of postprandial glucose (%PG) 31.4% ± 7.9% vs. 47.5% ± 8.6%, p=0.010, glucose peak 7.9 ± 0.3 vs. 8.9 ± 0.3 mmol/L, p=0.025 and 2h OGTT postprandial glucose (2hPG) 6.1 ± 0.3 vs. 6.7 ± 0.3 mmol/L, p=0.027. Glucose iAUC was not significantly different in the complex group, except for reductions in %PG 28.7% ± 8.2% vs. 43.4% ± 5.9%, p=0.012 after 50 mg dose and 27.7% ± 5.4% vs. 47.3% ± 7.2%, p=0.025 after 400 mg dose. The results suggest that Enzogenol® may have hypoglycaemic effects in healthy participants, especially those exhibiting monophasic shapes.

Keywords: New Zealand pine bark extract; Enzogenol®; proanthocyanidins; impaired glycaemic control; hypoglycaemic effects

4.1 Introduction

Type 2 diabetes mellitus (T2DM) is characterised by abnormally high blood glucose levels otherwise termed as hyperglycaemia [1]. It accounts for over 90% of diabetes diagnoses [2], and can result in significant morbidity and mortality from macrovascular and microvascular complications, such as retinopathy, nephropathy, and neuropathy [3-5].

Natural plant extracts have been increasingly explored as options to treat and manage impaired glycaemic control [6-9]. They are seen as a potential alternative to anti-diabetic medications that are associated with a range of adverse effects, including weight gain, risk of hypoglycaemia, and gastrointestinal discomfort [10]. One such natural extract is food-grade pine bark, obtained from the bark of pine trees in timber industries that otherwise would be discarded [11, 12]. Recent studies examining various sources of pine bark extracts have shown that pine bark possessed antioxidant properties, anti-inflammatory, neuro-protective, and anti-diabetic effects [11, 13, 14].

Naturally occurring polyphenols have been suggested to play a major role in the anti-diabetic properties exhibited by pine bark extracts, and proanthocyanidins are the main and most abundant bioactive components [11, 12, 15, 16]. The New Zealand pine bark extract, trade name Enzogenol®, is produced from *Pinus radiata* trees grown in New Zealand by a water-based extraction [11, 12]. The dry powder contains greater than 80% proanthocyanidins, 1–2% taxifolin, other flavonoids and phenolic acids, and some carbohydrates [12]. The proanthocyanidin content in Enzogenol® was also shown to be even higher than Pcynogenol®, a French Maritime pine bark produced from the outer bark of *Pinus pinaster* Ait. Subsp. *Atlantica* growing in the Southwest coastal region in France [16, 17].

There is increasing evidence showing that healthy individuals with normal glucose tolerance (NGT) may still eventually develop T2DM [18-22] depending on their patterns of postprandial glucose [23-28]. It is suggested that 20% of NGT individuals already have a certain degree of insulin resistance [25, 29-31], although their postprandial blood glucose may fall within the normal range.

Patterns of postprandial glucose obtained from the 2h oral glucose tolerance test (OGTT) have been extensively used in research to predict future T2DM in NGT individuals before its onset [32, 33]. Studies have shown that individuals with monophasic glucose curve shapes during a 2h OGTT, defined by having only one peak in the glucose curve, tended to have a heightened risk of T2DM [23-28]. On the other hand, those exhibiting biphasic or triphasic (complex) glucose curve shapes, defined by having more than one peak in the glucose curve,

are more likely to have a lower risk of T2DM [23-28]. Cross-sectional and longitudinal studies have observed that NGT individuals possessing monophasic glucose curve shapes, in contrast to those having complex shapes, tended to have a significantly higher glucose, insulin, C-peptide, free fatty acid, and visceral fat [24-26], with significantly reduced insulin sensitivity, a lack of compensatory first and second phase insulin secretion, and higher levels of insulin resistance [23-25, 27, 33].

Clinical studies have also revealed that depending on the degree of glucose tolerance in individuals defined by various glucose response indices including glucose curve shapes, they may either be responders or non-responders to a given intervention such as with natural plant extracts [34-37]. Krishnan and colleagues (2012) have also emphasised the importance of examining dietary interventions using an integrated physiological approach that stratified participants with varying glycaemic responses into subgroups in contrast to overall group responses [37].

To date, no human study has been conducted on the hypoglycaemic effects of Enzogenol® that was based on subgroup glycaemic responses to the intervention given. Hence, this present study was an exploratory study to first investigate the acute, dose-dependent, hypoglycaemic potential of Enzogenol® on healthy, NGT participants stratified into subgroups of glycaemic responses. The subgroups were based on their postprandial glucose curve shapes (monophasic vs. complex) after an OGTT at the control visit. Further to this, by stratifying participants according to their postprandial glucose curve shapes, we aimed to investigate if there was a difference in treatment outcome and dose response to Enzogenol® between the two groups. The study examined both the primary outcome measurement of glucose incremental area under the curve (iAUC) and secondary changes in glycaemic response indices, such as the percentage increment of postprandial glucose (%PG), the time to glucose peak, the glucose peak value, and 2h postprandial glucose at 120 min of OGTT (2hPG), to enhance the understanding of the potential hypoglycaemic effects of Enzogenol®. Any observed acute effects from this preliminary intervention with Enzogenol® in healthy population would help inform future studies that include investigating the impact of Enzogenol® over at least 8–12 weeks in both normo- and hyperglycaemic participants with worsening glucose intolerance, such as those with prediabetes or T2DM. Additionally, future studies can determine the effects of Enzogenol® on insulin secretion and sensitivity, as well as β-cell function (e.g., HOMA-IR, Matsuda index, and other measures), and explore the mechanistic action of Enzogenol® in improving blood glucose responses.

4.2 Methods

4.2.1 Study Population

The study was approved in March 2018 by the Massey University Human Ethics Committee (MUHEC) (ref: SOA 17/73). The clinical trial was registered retrospectively at anzctr.org.au (Australia New Zealand Clinical Trials Registry Number: ACTRN12619001571167). The study was conducted in accordance with the Declaration of Helsinki and all participants gave their informed consent prior to participating in the study.

Participants were recruited from Auckland, New Zealand using poster advertisements (**Appendix 2.1**) within the local university and community (March–December 2018). They were selected according to the following inclusion criteria: (i) healthy Body Mass Index (BMI) of 18.5–25.0 kg/m^2, (ii) aged 18–40 years, (iii) not suffering from any impaired glycaemic control (fasting blood glucose (FPG) < 5.5 mmol/L) and glycated haemoglobin A1c (HbA1c) < 40 mmol/mol (Cobas b 101 HbA1c test, CV 0.8–1.7%, Roche Diagnostics), (iv) not taking any forms of glucose-lowering medications or medications that may affect glucose metabolism, (v) free from any form of illnesses or chronic diseases. Participants were excluded from the study if they had any form of cardiovascular, metabolic diseases, digestive ailments, if they smoked, were pregnant or lactating, and if they had any known allergies to pine bark extract. Documents of recruitment can be found in **Appendix 2.2-2.6**.

4.2.2 Study Design

The study was an acute, placebo-controlled, single-blind, crossover, dose-response, exploratory study. The study required participants to come to the research facility at Massey University, Auckland, New Zealand for three separate visits with at least a 48 h washout period.

Briefly, at every visit, participants arrived at the facility after at least a 10 h overnight fast (water was allowed). The first study visit was a control visit whereby participants consumed a single placebo capsule containing microcrystalline cellulose. The second and third visits were treatment visits where patients consumed a single capsule of 50 and 400 mg of Enzogenol®, respectively. An OGTT was commenced at all three visits 20 min after consuming the capsule. Participants were given a bottle of 300 mL of glucose drink Carbotest (Fronine, Thermo Fisher Scientific, Victoria, Australia) containing 75 g of carbohydrates to consume within five minutes.

Capillary blood samples were obtained via finger pricking with a single use disposable lancet (Accu-Chek Safe T-Pro Plus) 20 min before the commencement of OGTT, and again at 0, 15, 30, 45, 60, 90, and 120 min during the OGTT. Blood glucose levels were immediately measured using a glucose meter (MediSense, Optium, Abbott, Auckland, New Zealand, 2.7–4.0% CV).

Participants were instructed to maintain a consistent diet without any dietary alterations throughout the duration of the study. They were also asked to abstain from alcohol and beverages such as teas, coffees, and energy drinks (both caffeinated and decaffeinated), all health supplements that might influence glucose metabolism, including any form of pine bark products, and strenuous activity during the 24h period before each visit. Compliance was checked at every visit by the researcher, including what they had eaten the previous day in order to ensure that they had been adhering to the dietary requirements regarding what they could eat or drink. Data collection form can be found in **Appendix 2.7**.

4.2.3 Treatments

Active capsules contained either 50 mg Enzogenol® plus 350 mg microcrystalline cellulose or 400 mg Enzogenol® prepared from the same batch of product. Placebo capsules contained 400 mg microcrystalline cellulose, which is commonly used as a filler in placebo capsules in clinical studies [38]. Both were supplied by ENZO Nutraceutical Limited (Paeroa, New Zealand). The doses chosen for this study were based on previous studies investigating the impact of pine bark (French Maritime) on glycaemic control in people with diabetes [39-41]. The doses chosen reflected the low and high doses used in previous studies with the focus on examining the acute hypoglycaemic impact of Enzogenol® in healthy participants. The doses selected represented concentrations with no known toxicity [12]. The identical looking capsules were of opaque white appearance, concealing the visibility of the contents, and were produced to the standards of dietary supplement preparations and quality control in New Zealand.

4.2.4 Parameters of Glycaemic Response during the OGTT

The shape of the control glucose curve of each participant was determined using calculations employed by Tschritter et al. (2003) [23], Briefly, monophasic, biphasic, and triphasic curves were defined as $Gluc_{120}$-$Gluc_{90}$ <0.25 mmol/L, $Gluc_{120}$-$Gluc_{90}$ >0.25 mmol/L and $Gluc_{90}$-$Gluc_{60}$ >0.25 mmol/L, respectively. Participants exhibiting either a biphasic or

triphasic glucose curve shape were grouped together as having a complex glucose curve shape.

The primary outcome, iAUC of postprandial glucose, was calculated during the OGTT from 0 to 120 min using the trapezoidal rule [42].

The %PG was defined by the percentage increment of 2hPG with respect to fasting blood glucose (FBG), using the formula [(2hPG-FBG)/FBG] × 100 [30].

The time to glucose peak was defined as the time point on the OGTT when the glucose level was highest. For complex glucose curve shapes (biphasic or triphasic), the time of the first glucose peak was considered [43].

The glucose peak value and the 2hPG were also recorded.

4.2.5 Statistical Analysis

A sample size calculation was performed on the change in postprandial capillary blood glucose based on a previous study conducted by our group on the acute responses of several plant extracts [44]. A minimum sample size of ten was required to detect a difference in postprandial glucose levels. However, to allow for potential withdrawals and the stratification of participants into two groups with either monophasic or complex glucose curve shapes, 25 participants were recruited.

A one-way factorial repeated measures ANOVA (95% confidence interval) was conducted to examine the effects of Enzogenol® on postprandial blood glucose and glucose response indices in participants within each glucose curve shape classification. Statistical analysis was done comparing each dose (50 mg and 400 mg) with control.

Monophasic and complex groups were statistically compared with an Independent Student two-tailed *t*-test assuming equal variance. Analyses were performed with the SPSS software version 25 (IBM Corporation, New York, NY, USA). The results were reported as mean ± S.E.M.

4.3 Results

Twenty-five healthy participants (ten men and fifteen women, mean age 24.8 ± 0.8 years) were recruited into the study. All participants completed both the control and the 50 mg Enzogenol® visit, and 20 also completed the 400 mg Enzogenol® study visit. The demographics of this group and the comparison between the monophasic and complex groups are reported in **Table 4.1**. All participants were considered healthy based on their baseline

data of mean BMI (21.2 ± 0.4 kg/m^2), FBG (4.4 ± 0.1 mmol/L), HbA1c (33 ± 0 mmol/mol), blood pressure (systolic 109 ± 2, diastolic 67 ± 1 mmHg), and lipid profiles [45]. There was no significant difference in baseline characteristics between the monophasic and complex groups (p>0.05).

Table 4.1 Baseline characteristics of participants in the Pine Bark study

Characteristics	Mean ± SEM (All)	Range (Min to Max)	Mean ± SEM (Monophasic Group)	Mean ± SEM (Complex Group)	*p* value (Monophasic vs. Complex Shape)
N	25	NA	12	13	-
Gender (M/F)	10/15	NA	3/9	7/6	-
Age (years)	24.8 ± 0.8	20–33	25.4 ± 1.1	24.2 ± 1.2	0.44
BMI (kg/m^2)	21.2 ± 0.4	18.1–25.3	21.2 ± 0.6	21.2 ± 0.5	1.00
FBG (mmol/L)	4.4 ± 0.1	3.6–5.2	4.5 ± 0.1	4.3 ± 0.1	0.55
HbA1c (mmol/mol)	33 ± 0	29–38	33 ± 1	34 ± 1	0.24
SBP (mm Hg)	109 ± 2	89–133	112 ± 4	107 ± 2	0.30
DBP (mm Hg)	67 ± 1	47–77	66 ± 2	68 ± 2	0.45
TC (mmol/L)	4.09 ± 0.12	3.06–5.27	4.07 ± 0.13	4.10 ± 0.20	0.89
TG (mmol/L)	1.15 ± 0.13	0.56–3.71	1.30 ± 0.25	1.02 ± 0.11	0.30
HDL-C (mmol/L)	1.50 ± 0.08	0.98–2.49	1.47 ± 0.11	1.54 ± 0.11	0.67
LDL-C (mmol/L)	2.06 ± 0.09	1.23–2.93	2.01 ± 0.13	2.10 ± 0.12	0.60
Non-HDL-C (mmol/L)	2.58 ± 0.09	1.88–3.32	2.60 ± 0.13	2.57 ± 0.13	0.87
TC/HDL-C ratio	2.82 ± 0.10	1.8–4.1	2.89 ± 0.17	2.75 ± 0.12	0.49

Values are means (± SEM). Abbreviations: BMI: body mass index; FBG: fasting blood glucose; HbA1c: glycated haemoglobin A1c; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

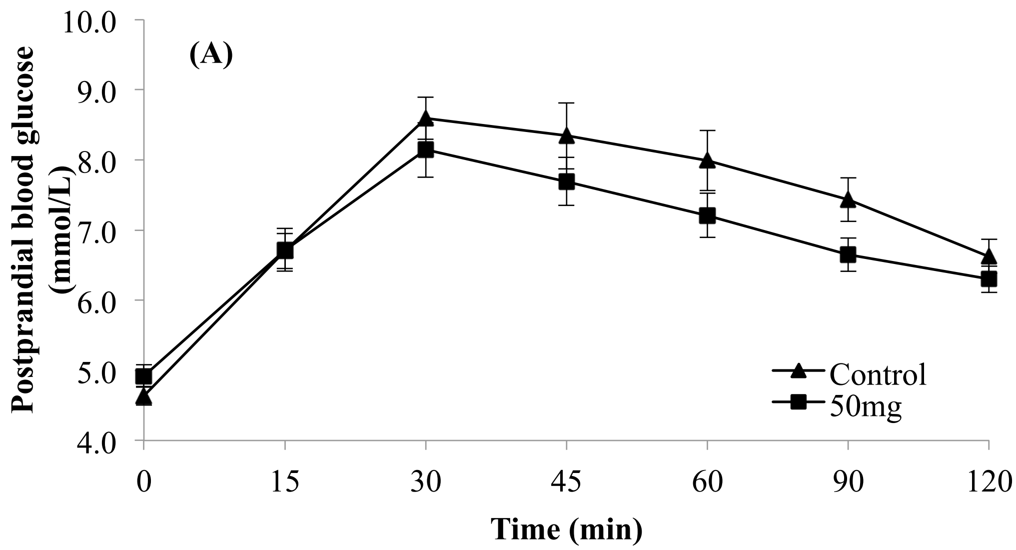
The overall data obtained from all participants was first analysed before the stratification of participants into two groups. There was no significant reduction in the primary outcome mean glucose iAUC observed between control and intervention with 50 mg of Enzogenol® (239.8 ± 19.2 vs. 276.8 ± 23.9 mmol/L·min, 13.4% reduction, p=0.123), but a significant reduction between the control and 400 mg of Enzogenol® (235.7 ± 16.5 vs. 299.5 ± 26.9 mmol/L·min, 21.3% reduction, p=0.016). There was no significant dose response between the two doses 50 and 400 mg (p=0.685).

The participants were then classified into two distinct groups based on their glucose curve shapes at the control visit, either monophasic or complex group. Participants with monophasic shapes were shown to have a significantly higher mean glucose iAUC (p=0.015) and glucose peak values (p=0.021) than participants with complex shapes at the control visit, indicating the presence of two distinct groups with dissimilar glycaemic control. The effects of Enzogenol® on postprandial glucose excursion differed depending on the type of postprandial glucose curve shapes participants exhibited at the control visit (**Figures 4.1 and 4.2**). In **Figure 4.1**, involving participants with monophasic shapes, a consistent trend of reduced glucose levels was observed with both doses of Enzogenol® compared to the control, although significantly lower glucose levels at 30, 60, and 120 min were only seen in the intervention with a 400 mg dose. **Figure 4.2** illustrated more heterogeneous glucose excursions involving participants having complex shapes with no obvious trend after consuming both doses of Enzogenol®.

In the monophasic group, 33.4% of participants shifted from monophasic to having complex shapes (16.7% with biphasic and 16.7% with triphasic) after consuming 50 mg of Enzogenol®, and 54.6% of participants shifted to having complex shapes (18.2% with biphasic and 36.4% with triphasic) after consuming 400 mg of Enzogenol®.

With reference to **Table 4.2**, Enzogenol® significantly reduced mean glucose iAUC in participants with a monophasic shape compared to control at both 50 mg (241.3 ± 20.2 mmol/L·min vs. 335.4 ± 34.0 mmol/L·min, 28.1% reduction, p=0.034) and 400 mg of Enzogenol® (249.3 ± 25.4 mmol/L·min vs. 353.6 ± 31.5 mmol/L·min, 29.5% reduction, p=0.012). However, there was no significant dose response observed between 50 and 400 mg of Enzogenol® within the monophasic group (p=0.881), suggesting that a smaller dose of 50 mg was equally effective in reducing mean glucose iAUC of participants. In contrast, neither dose of Enzogenol® significantly reduced the glucose iAUC in the complex group.

Mean %PG was reduced in both monophasic and complex groups compared to control. Treatment with 400 mg of Enzogenol® in the monophasic group significantly reduced mean %PG compared to control (31.4% ± 7.9% vs. 47.5% ± 8.6%, 33.9% reduction, p=0.010). No significant reduction in mean %PG was observed in this group after consuming 50 mg Enzogenol®. In the complex group, compared to control, the mean %PG was significantly reduced by both 50 mg (28.7% ± 8.2% vs. 43.4% ± 5.9%, 33.8% reduction, p=0.012) and 400 mg of Enzogenol® (27.7% ± 5.4% vs. 47.3% ± 7.2%, 41.4% reduction, p=0.025).



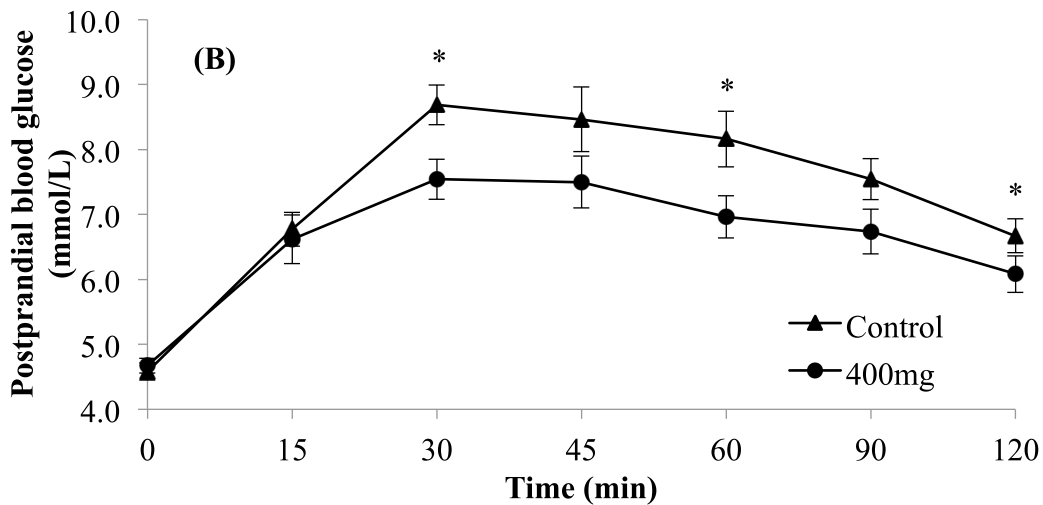


Figure 4.1 Mean postprandial glucose (±SEM) of participants with a monophasic glucose curve shape during the control and treatment visits with (A) 50 and (B) 400 mg of Enzogenol®. * *p*<0.05 for 400 mg Enzogenol® compared to control.

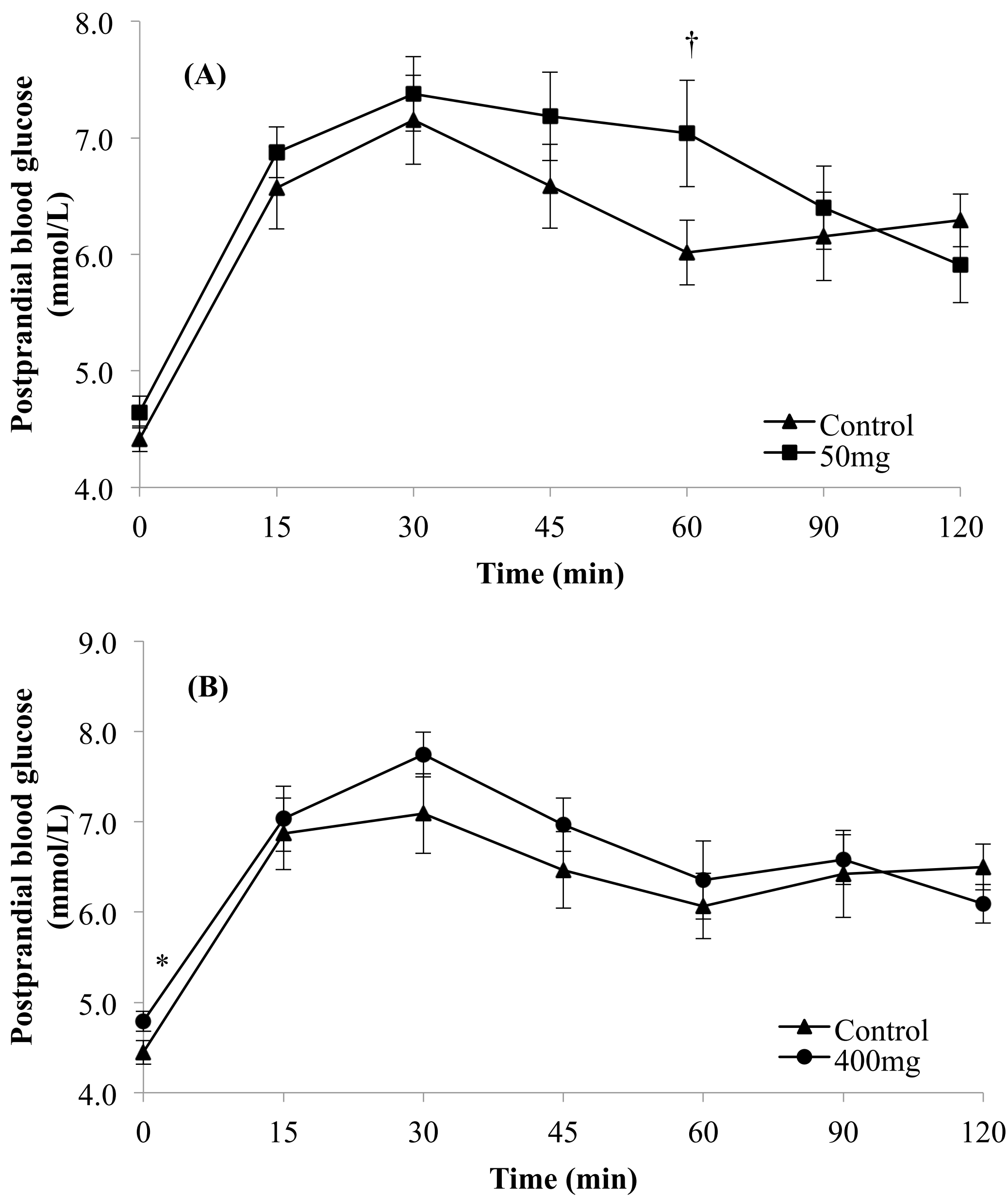


Figure 4.2 Mean postprandial glucose (±SEM) of participants with a complex glucose curve shape during the control and treatment visits with (A) 50 and (B) 400 mg of Enzogenol®. † *p*<0.05 for 50 mg Enzogenol® compared to control, * *p*<0.05 for 400 mg Enzogenol® compared to control.

Table 4.2 Parameters of glycaemic response in participants classified as having either a monophasic or complex glucose curve shape at control visit and treatments with 50 and 400 mg of Enzogenol®

Parameters of Glycaemic Response	Monophasic Group						Complex Group					
	Control Paired to 50 mg of Enzogenol®	50 mg of Enzogenol®	*p*	Control Paired to 400 mg of Enzogenol®	400 mg of Enzogenol®	*p*	Control Paired to 50 mg of Enzogenol®	50 mg of Enzogenol®	*p*	Control Paired to 400 mg of Enzogenol®	400 mg of Enzogenol®	*p*
n	12		-	11		-	13		-	9		-
Gender (M/F)	3/9		-	3/8		-	7/6		-	5/4		-
Mean glucose iAUC (mmol/L·min)	335.4 ± 34.0 †	241.3 ± 20.2	0.034 *	353.6 ± 31.5 ‡	249.3 ± 25.4	0.012 *	222.7 ± 26.6 †	238.4 ± 32.7	0.392	233.5 ± 36.4 ‡	219.1 ± 19.5	0.614
Mean %PG	44.9 ± 8.3	28.9 ± 3.8	0.083	47.5 ± 8.6	31.4 ± 7.9	0.010 *	43.4 ± 5.9	28.7 ± 8.2	0.012*	47.3 ± 7.2	27.7 ± 5.4	0.025 *
Mean time to glucose peak (min)	35.0 ± 2.8	32.5 ± 2.5	0.504	35.5 ± 3.0	32.7 ± 4.0	0.441	28.8 ± 2.7	28.8 ± 4.0	1.000	28.3 ± 3.0	26.7 ± 4.9	0.729
Mean glucose peak value (mmol/L)	8.8 ± 0.3†	8.3 ± 0.4	0.177	8.9 ± 0.3‡	7.9 ± 0.3	0.025 *	7.7 ± 0.3 †	8.2 ± 0.4	0.315	7.8 ± 0.3 ‡	8.0 ± 0.3	0.531
Mean 2hPG (mmol/L)	6.6 ± 0.2	6.3 ± 0.2	0.171	6.7 ± 0.3	6.1 ± 0.3	0.027 *	6.3 ± 0.2	5.9 ± 0.3	0.149	6.5 ± 0.3	6.1 ± 0.2	0.224

* Significant difference between control and 50 or 400 mg of Enzogenol® within each glucose curve shape group ($p<0.05$); † Significant difference in control visit values between monophasic and complex glucose curve shape groups at 50 mg of Enzogenol® ($p<0.05$); ‡ Significant difference in control visit values between monophasic and complex glucose curve shape groups at 400 mg of Enzogenol® ($p<0.05$); Abbreviations: iAUC: incremental area under the curve of postprandial glucose; %PG: percentage increment of postprandial glucose; 2hPG: 2h postprandial glucose at 120 min of the oral glucose tolerance test (OGTT).

There was no significant difference in mean time to glucose peak observed between control and all treatments in monophasic and complex groups. Most participants had their glucose peaking at 30 min during the OGTT, with a higher percentage of participants in the complex group having an earlier glucose peak at 15 min compared to monophasic group.

Within the monophasic group, a reduction in the mean glucose peak value was observed following both 50 and 400 mg of Enzogenol®, with the 400 mg dose being statistically significant (7.9 ± 0.3 vs. 8.9 ± 0.3 mmol/L, 11.2% reduction, p=0.025) (**Figure 4.1**). There was no significant difference observed between control and treatments within the complex group.

Mean 2hPG was significantly reduced in the monophasic group treated with 400 mg of Enzogenol® (6.1 ± 0.3 vs. 6.7 ± 0.3 mmol/L, 8.9% reduction, p=0.027) (**Figure 4.1**). No significant changes were observed with 50 mg or in the complex group.

There was a lack of significant dose response observed between the two doses 50 and 400 mg in both the monophasic and complex groups in the primary and secondary outcome measurements (p>0.05).

4.4 Discussion

Type 2 Diabetes Mellitus is a worldwide epidemic with an increasing number of people not adhering to a healthy diet and an active lifestyle as key drivers in the increased risk of developing T2DM, although other determining factors such as genetics, environment and socioeconomic factors may play a part [5, 46]. Because the progression of normoglycaemia towards impaired glycaemic control is a continuum, and even healthy, NGT individuals have been shown to have a certain degree of insulin resistance [25, 29-31], it is becoming increasingly important to be able to classify individuals based on the varying degrees of dysglycaemia.

In the present study, a significant reduction in the primary outcome mean glucose iAUC was only seen for the 400 mg dose of Enzogenol® (p=0.016). However, when the participants were stratified by their baseline postprandial glucose curve shapes, it was shown that it was effective in reducing the postprandial glucose iAUC at both dose levels in participants with monophasic shapes. This group could be termed as responders as they responded well to the intervention by demonstrating improved glycaemic responses, such as a significantly reduced postprandial glucose iAUC, with additional improvements in other glucose response indices at the higher dose, 400 mg. This outcome was in contrast to participants with complex shapes

who may then be termed as non-responders, as no significant improvements in the primary outcome glucose iAUC and other glucose response indices measured, except for %PG were evident.

To the best of our knowledge this is the first study to report on the glycaemic-lowering properties of New Zealand pine bark extract in humans. Prior to this study, a diabetic mouse model fed with Enzogenol® was reported to improve diabetes-related biomarkers with a reduction in HbA1c, insulin, and glucagon levels, and an elevation of hepatic AMP-activated protein kinase (AMPK) activity [47]. Pcynogenol®, a French maritime pine bark containing similar types of phenolic content but in different quantities compared to Enzogenol®, has been more extensively studied. Several chronic human trials have examined the impact of Pcynogenol® in doses of 50–300 mg on glycaemic responses, such as FBG and HbA1c in T2DM participants [39-41]. All three studies showed a significant reduction in FBG and two studies showed a significant reduction in HbA1c. These studies elucidated the potential hypoglycaemic effects of pine bark, although they were conducted in T2DM participants, which differed from the present study with healthy participants.

The present study has employed the use of various glycaemic response indices during the 2h OGTT in relation to postprandial glucose curve shapes to investigate if Enzogenol® would have an impact on various indicators of glycaemic control.

A study conducted by Schianca et al. (2010) elucidated that normoglycaemic individuals with lower %PG closer to FBG possessed higher insulin sensitivity and decreased insulin secretion compared to individuals with higher %PG [30]. Treatment with 400 mg of Enzogenol® in the monophasic group and both doses, 50 and 400 mg, in the complex group significantly reduced %PG compared to control. The reduction in %PG could indicate the increased rate in returning postprandial blood glucose back to baseline FBG after two hours in both monophasic and complex groups with the consumption of Enzogenol®, which would suggest that there was a potential acute improvement in glycaemic control [48].

Research has shown that individuals exhibiting a monophasic glucose curve shape compared to those with a complex glucose curve shape (biphasic or triphasic) had a delayed rise in glucose with a later glucose peak, a higher glucose peak value, and a higher 2h postprandial glucose value, and are more likely to be at an increased risk of diabetes due to suboptimal glycaemic control [24, 43]. Our observations agree with other studies where individuals with monophasic shapes exhibited higher postprandial glucose concentrations [23-28]. A delay in glucose peak time often indicates reduced insulin sensitivity and secretion and a rise in glucose typically observed in T2DM [49]. A time to glucose peak above 30 min

was indicated to be a reproducible independent indicator of impaired glycaemic control [43, 50]. Participants in the present study had mean glucose peaks occurring within 30 min on average for the complex group at control and with both treatments with Enzogenol® (*p*>0.05). In contrast, the mean time to glucose peak in the monophasic group was slightly above 30 min for both control and treatments with Enzogenol®, although the time to glucose peak was shorter compared to control (*p*>0.05).

The level of glucose peak has been associated with increased oxidative stress corresponding to the level of postprandial glucose toxicity [51]. Hulman et.al. concluded that a higher than normal glucose peak is more predictive of abnormality with insulin sensitivity than an absolute 2hPG value [52]. A recent cohort study further elucidated that individuals with a higher intermediate glucose value and a lower 2hPG value were associated with a higher risk of future diabetes than those with a higher 2hPG but a lower intermediate glucose value [53]. The present study found that 400 mg of Enzogenol® was able to significantly reduce the glucose peak value compared to control in the monophasic group (*p*=0.025).

In the San Antonio Metabolism (SAM) study, so-called "normal" glucose-tolerant individuals having 2hPG values within the range of 6.7 to 7.8 mmol/L were found to exhibit a 40–50% decrease in β-cell function compared to individuals with 2hPG lesser than 5.6 mmol/L [31]. Participants in the present study had mean 2hPG values that were lower than the at-risk range in all treatments except for participants in the monophasic group. Monophasic participants in the present study had a mean 2hPG value of 6.6 ± 0.2 mmol/L (this being obtained from capillary blood sample instead of plasma, as in the SAM study), which was close to the stated range indicative of a certain degree of β-cell dysfunction. However, this higher 2hPG value was diminished with a subsequent treatment with Enzogenol®.

One of the merits of the study was the use of a robust crossover design where participants were their own control and underwent each of the treatments, and therefore a smaller sample size was required for the same level of significance. To our knowledge, this present study was one of the first intervention studies to classify normoglycaemic participants based on their degrees of impaired glycaemic control dependent on their glucose curve shapes, with the inclusion of useful glycaemic indices to determine if Enzogenol® was effective in improving glycaemic responses. Furthermore, the potential modification of monophasic shapes into complex shapes with the consumption of Enzogenol® was also examined. It was observed that about 33% of the monophasic participants changed to having complex shapes after consuming 50 mg of Enzogenol®, and about 55% of monophasic

participants had complex shapes after consuming 400 mg of Enzogenol®. This might potentially alter T2DM risk based on the glucose shapes. This observation was in line with Manco et al. (2017), who concluded that individuals who persisted in having monophasic shapes and those who switched from having biphasic to monophasic shapes over a prolonged period had an increased risk of impaired glycaemic control [54]. A longer-term study is warranted to examine the persistence in shape alteration in relation to changes in glucose metabolism with the consumption of Enzogenol®.

Nevertheless, the study was not without limitations, which may include determining the glucose curve shapes based on the results of a single OGTT. Although, OGTT has been known as a sensitive test to detect mild disturbance in glucose metabolism and disposal [55], its reproducibility has been questioned. This was due mainly to differences in the rate of glucose absorption and insulin responses with varying degrees of glucose and insulin oscillations in individuals [56] and intra-individual variations [57-59], although one study found it to be repeatable in healthy individuals [60]. Hence, having repeated OGTT at baseline may improve reproducibility.

The classification of individuals into different glucose curve patterns might be simple and useful in an acute study with a smaller sample size, such as in the present study, but limitations may include the possible misclassification of individuals, especially if they exhibit heterogeneous glucose curves. However, we have classified our participants based on the calculations by Tschritter et al. (2003) [23] and verified with each postprandial glucose curve of each participant at the control visit to ensure there was no misclassification.

This study also only looked at postprandial glycaemic responses. Future studies should include insulin or C-peptide measurements to further our understanding of the effect of Enzogenol® consumption on insulin secretion, insulin sensitivity, and β-cell function (e.g., HOMA-IR, Matsuda index, and other measures). Future work may also explore the underlying mechanistic actions of Enzogenol® in its glucose modulating effects in order to optimise on the dosage effective for lowering postprandial blood glucose. More investigation is warranted regarding the physiological metabolism of Enzogenol® in humans through characterising phenolic metabolites from biological samples to investigate how the extract is being metabolised, absorbed, transformed, and excreted from the body. Future studies should also measure chronic glycaemic responses to Enzogenol® over at least 8–12 weeks in both normo and hyperglycaemic participants. Future studies could also look into how Enzogenol® can be incorporated into the diet, such as the effective dose, frequency, and sequence of

consumption that helps to maintain a healthy glycaemic control and extend its functionality to individuals with varying degrees of impaired glycaemic control.

4.5 Conclusions

The present study in healthy participants shows that Enzogenol® has hypoglycaemic effects, however, there was significant variation in inter-individual response, which appears to be driven by dissimilar individual glycaemic profile, with participants having monophasic glucose curve shapes showing greater improvements in postprandial glucose responses. Compared to healthy participants with complex glucose curve shapes, monophasic participants showed a significant improvement in mean glucose iAUC for both 50 and 400 mg doses of Enzogenol®, with a further reduction in glucose peak value, 2hPG, and %PG with 400 mg of Enzogenol® compared to control. In contrast, glycaemic responses in participants with complex glucose curve shapes were not altered with the ingestion of Enzogenol® except for significant reduction in %PG values. Although Enzogenol® appears to show hypoglycaemic potential, future studies are warranted to examine the effect on other measures, such as insulin, and in other population groups, such as those with prediabetes or T2DM.

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Chapter 5

Hypoglycaemic effects of antioxidant-rich plant extracts on postprandial glycaemic responses in participants with prediabetes (GLARE study): A single-blind, placebo-controlled, crossover study

This chapter presents the study outcomes examining the effect of three plant extracts: grape seed, rooibos tea and olive leaf on glycaemic control in participants with prediabetes. This chapter has been presented in manuscript format and prepared for submission to the *Journal of Nutrition*.

Abstract

Background: Prediabetes is a state of intermediate hyperglycaemia and is considered a critical stage for intervention to potentially reduce the risk of type 2 diabetes mellitus (T2DM) development. Dietary polyphenols such as plant extracts may help in glycaemic control.

Objective: Investigate the acute effect of grape seed, rooibos tea, and olive leaf extracts on postprandial blood glucose and insulin responses in participants with prediabetes.

Methods: An acute, single-blind, placebo-controlled, crossover study (ACTRN12617000837325) where extracts of grape seed, rooibos tea and olive leaf, and placebo were given on separate occasions with an oral glucose tolerance test to 19 participants. Measures of glycaemic indices were performed and data analysed using linear mixed model for repeated-measures.

Results: Participants were classified into either a healthier or less healthy subgroup based on the time to peak of glucose and insulin. In the less healthy subgroup, all extracts showed improvements in glycaemic responses. Compared to placebo, grape seed reduced glucose levels such as incremental area under the curve of glucose, $iAUC_{glucose}$ (p=0.016, 21.9% reduction), 2h postprandial glucose, 2hPG (p=0.034, 14.7% reduction) and metabolic clearance rate of glucose, MCR (p=0.016, 16.7% increase), and improved indices of insulin such as 2h postprandial insulin, 2hPI (p=0.029, 22.4% reduction) and Stumvoll overall insulin sensitivity index, $ISI_{overall}$ (p=0.028, 15.0% increase). Rooibos tea extract significantly improved β-cell function as demonstrated by the increased oral disposition index, DI (p=0.031, 32.4% increase) and showed trend towards improvement for insulin-secretion-sensitivity index-2, ISSI-2 (p=0.06, 19.9% increase) compared to placebo. Olive leaf extract significantly increased insulin secretion by an increased incremental area under the curve, $iAUC_{insulin}$ (p=0.040, 16.7% increase) and showed trend towards improvement for indices of insulin sensitivity such as Stumvoll first phase insulin sensitivity index, ISI_{first} (p=0.08, 17.8% increase), Stumvoll second phase insulin sensitivity index, $ISI_{second,}$ (p=0.06, 15.6% increase), and insulinogenic index, IGI_{30} (p=0.08, 27.8% increase) compared to placebo.

Conclusions: Grape seed, rooibos tea and olive leaf extracts demonstrated acute hypoglycaemic benefits in individuals with prediabetes with a less healthy metabolic profile. A chronic study on the plant extracts is warranted to determine their longer-term impact on prediabetes.

5.1 Introduction

Prediabetes is characterised by isolated impaired fasting glucose (IFG) or isolated impaired glucose tolerance (IGT), or a combination of both (IFG/IGT) [1]. It is the intermediate state of hyperglycaemia with blood glucose levels above normal but below the threshold to be classified as type 2 diabetes mellitus (T2DM) [2]. It is estimated that globally, 374 million (ages 20-79 years) (7.5%) people have prediabetes and the global prevalence is projected to be 548 million (8.6%) by 2045 [3]. Prediabetes has an annual conversion rate of 5-10% to T2DM [2, 4]. According to an American Diabetes Association expert panel, up to 70% of individuals with prediabetes will eventually develop T2DM [5].

Whilst there is unequivocal evidence that many individuals with prediabetes are able to prevent the conversion to T2DM through lifestyle and diet modification [6-9], research is scarce regarding the use of complementary medicines and natural plant extracts to treat prediabetes. Studies of dietary polyphenols from natural plant sources have shown that some could have hypoglycaemic potential [10-17]. Proposed mechanisms by which they can improve glucose metabolism include the inhibition of digestive enzymes (α-amylase and α-glucosidase) and glucose transporters, activation of glucokinase, increase in adiponectin levels, and elevation of incretin levels to stimulate glucose-dependent insulin secretion [10, 11, 18, 19]. Hence, polyphenol-rich plant extracts might be a useful adjunct in the preventing progression of prediabetes to T2DM.

Clinical trials conducted on healthy individuals and those with metabolic syndrome and T2DM have shown equivocal results regarding the consumption of grape seed [20-29], rooibos tea [29, 30], and olive leaf extracts [31-38] likely due to concomitant medications, small sample sizes, as well as the plant extract sources, degree of purification and varying concentrations of extract administered in the trials. More importantly the inherent differences in metabolic profiles of participants might have likely contributed to the differences observed in the studies.

It is increasingly recognised that individuals with prediabetes are not a homogenous group and that differences in metabolic profiles identified as having different postprandial glucose shapes [39-44], postprandial glucose indices [40, 45-49], and patterns of insulin concentrations [50-52] have been associated with varying degrees of risk towards T2DM. Therefore stratification based on the different metabolic profiles is important in order to elucidate the impact of intervention on both responders and non-responders [53-55]. This

would allow the opportunity for optimisation of nutritional interventions for each specific metabolic profile.

More recently, Takahashi and colleagues (2018) examined the stratification based on the time to peak in glucose and insulin responses to differentiate between heterogeneous metabolic profiles of participants [56]. They classified 62 healthy women into four groups: (1) normal type, (2) insulin-late type, (3) insulin- and glucose-late type, and (4) insulin-very late type [56]. They observed that whilst the first two groups had no significant differences in homeostatic model assessment-insulin resistance (HOMA-IR), homeostatic model assessment-beta-cell function (HOMA-β), area under the curve of glucose ($AUC_{glucose}$) and area under the curve of insulin ($AUC_{insulin}$), (3) and (4) groups showed distinctly delayed insulin secretory responses and elevated $AUC_{glucose}$. Other studies have corroborated that a delay in glucose peak time has been used as a reproducible index for T2DM risk and a value of above 30 min indicates presence of insulin resistance and poorer glycaemic control [46, 47, 57]. Insulin patterns such as delayed insulin response have been linked to defects in β-cell function and diminished insulin sensitivity in individuals and are associated with an increased risk of developing T2DM [50, 52, 58-63]. Therefore it was useful to employ this stratification in the secondary analysis of this present study to explore the response to intervention of participants with prediabetes but exhibiting varying metabolic profiles.

In our previous work we have shown that antioxidant-rich plant extracts such as the grape seed and rooibos tea extracts had helped to improve acute postprandial blood glucose response by 25-40% in healthy participants [29]. A chronic study conducted by De Bock et al. (2013), also demonstrated that with olive leaf insulin sensitivity and pancreatic β-cell responsiveness were significantly improved in normoglycaemic, overweight men [33]. To date no study has investigated the hypoglycaemic potential of these plant extracts in a group with prediabetes.

The present GLARE (Glucose Lowering Antioxidant-Rich plant Extracts) study aimed to examine the acute impact of grape seed, rooibos tea and olive leaf extracts on postprandial blood glucose response in individuals with prediabetes living in New Zealand.

5.2 Materials and Methods

5.2.1 Study participants

Participants were recruited from Auckland, New Zealand using poster advertisements (**Appendix 3.1**) within the local university and community (August 2017-May 2019). They

were selected if they met all inclusion criteria at screening: (1) having prediabetes with a glycated haemoglobin A1c (HbA1c) value between 41-49 mmol/mol (5.9-6.6%) or a lower HbA1c of 38-40 mmol/mol (5.6-5.8%) and having at least one of the T2DM risk factors (overweight or obese, high blood pressure, had prediabetes before, and family history of T2DM or cardiovascular disease (CVD)), (2) not taking any form of glucose-lowering medications or medications having an iatrogenic nature that may influence glucose metabolism, particularly β-blockers for cardiovascular diseases and thiazide diuretics for hypertension, (3) no known pancreatic, hepatic, renal or digestive impairments that may alter glucose metabolism or metabolism of plant extracts, (4) no known allergies to the plant extracts under study, and (5) not smoking. Interested individuals initially completed an online screening questionnaire (**Appendix 3.2**) on T2DM risk (Ministry of Health, New Zealand) [64] before potential participants were invited into Massey University Human Nutrition Research Centre (MUHNRC) in Auckland for more in-depth screening. Measurements of their body composition including height, weight, body fat and muscle mass (Bioelectrical Impedance Analysis, InBody 230, Biospace co., Ltd.), waist and hip circumference, blood pressure (Omron, HEM-907, Omron Healthcare Co., Ltd.), HbA1c (Cobas b 101 HbA1c test, CV 0.8-1.7%, Roche Diagnostics) were taken. Documents of recruitment can be found in **Appendix 3.3-3.7**.

All participants gave their informed consent for inclusion before they participated in the study (**Appendix 3.6**). The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Massey University Human Ethics Committee (MUHEC) (ref: 17/STH/82). The clinical trial was registered prospectively at the Australian New Zealand Clinical Trials Registry and accessible at http://www.anzctr.org.au/ (ACTRN12617000837325).

5.2.2 Study design

The study was an acute, single-blind, placebo-controlled, crossover study involving 19 participants. The study involved four visits to where participants were required to fast for at least ten hours except water before each visit.

During the control visit, participants were given two placebo capsules to swallow with a few sips of water. This was followed by a 2h oral glucose tolerance test (OGTT) involving consuming a 300 mL glucose drink containing 75 g of glucose (Carbotest, Fronine, Thermo Fisher Scientific, Australia) within five minutes, and repeated measures of blood glucose

during the following two hours. The subsequent three visits involved consuming one of the three plant extracts in the following order: grape seed, rooibos tea, and olive leaf on separate visits with at least a washout period of one week between visits. The OGTT procedure was repeated for each visit. All three plant extracts were matched for their antioxidant capacity and administered in standardised capsule form. Antioxidant capacity has been shown in previous research to correlate with total phenolics [65, 66], and hence a good indicator of overall phenolic composition within each plant extract.

At each visit baseline venous blood samples were taken at 10 min prior to the consumption of the capsules, followed by another baseline sampling at t=0 min before the commencement of the OGTT. Further blood samples were obtained at t=15, 30, 45, 60, 90, and 120 min. Blood draws were done by having the participant lie in a supine position and cannulating the antecubital fossa region of the arm. Blood samples were collected into heparin and serum tubes and centrifuged at 3,500 rpm for 15 min at 4°C. Blood collected in heparin tubes were immediately centrifuged after collection, whilst blood collected in serum tubes were left to coagulate for at least half an hour before centrifuging. They were then aliquot into Eppendorf tubes and stored at -80°C until analysis.

At each trial visit, all participants were checked for dietary and lifestyle compliance, such as having fasted at least 10 hours prior to the visit, keeping their diet constant, no strenuous physical activity, no alcohol, no caffeinated tea or coffee formulations, no health supplements 24 hours prior to the visit, and no consumption of active plant extracts (grape seed, rooibos tea, olive leaf) throughout the duration of the study. Data collection form can be found in **Appendix 3.8.**

5.2.3 Treatments

All the plant extract samples were obtained commercially. Grape seed extract (*Vitis vinifera*) (Nutra-Life, Vitaco Health (NZ) Ltd.) was produced with 40 g of dry grape seed and 10 g of fresh grape seed per capsule and contained 640 mg of procyanidins in the concentration given to participants. Rooibos tea extract (*Aspalathus linearis*, E2CCJ) (Rooibos Limited, South Africa) contained at least 485 mg of total polyphenols in the concentration given to participants. Olive leaf extract (*Olea europaea*) (Comvita NZ Ltd) was made from 3.5 g of fresh leaf suspended in olive oil per capsule and contained 264 mg of oleuropeins in the concentration given to participants. The plant extracts were commercialised products and had no known adverse toxic effects associated with them or similar products as the levels consumed [67-69]. All extracts were prepared according to

good manufacturing practice (GMP). The placebo capsule was made of 500 mg microcrystalline cellulose.

Sample extracts of grape seed, rooibos tea, and olive leaf were sent to Callaghan Innovation (Wellington, New Zealand) to determine their total antioxidant capacity (TAC) using the Oxygen Radical Absorbance Capacity (ORAC) assay. Following a previous study, but with some modifications [70], the ORAC assay was carried out with each sample of plant extract dissolved in a ratio of ethanol:water mixture (70:30, v/v) and their TAC determined.

Extracts of grape seed, rooibos tea, and olive leaf were matched at similar TAC content at 8,499 trolox equivalent (TE) µmol, 8,496 TE µmol, and 9,152 TE µmol, respectively, with concentrations based on a previous study [29]. Due to the nature of the olive leaf extract that was suspended in oil in a soft-gel and packed in a hard casing, contents could not be modified to obtain closer TAC values with grape seed and rooibos tea extracts.

5.2.4 Measures of glucose and insulin responses during the OGTT

The primary outcome of this study was incremental area under the curve of glucose ($iAUC_{glucose}$). The mean iAUC of the blood glucose ($iAUC_{glucose}$) and insulin ($iAUC_{insulin}$) were analysed using the trapezoidal rule [71, 72]. Other indices of glucose such as 2h postprandial glucose value (2hPG), and glucose peak time were measured. Time to glucose peak was defined as the time point on the OGTT when the glucose level was highest. If two equal peaks occurred during the OGTT, the earlier peak was considered as the peak. Similarly, $iAUC_{insulin}$, 2h postprandial insulin (2hPI), and insulin peak time were calculated. Insulin sensitivity indices such as the Matsuda Insulin Sensitivity Index (ISI/M) [73], Stumvoll overall insulin sensitivity ($ISI_{overall}$) [74], and Oral Glucose Insulin Sensitivity (OGIS) [75] were calculated. The OGIS was calculated using a web-based computerised formula assessable at: http://webmet.pd.cnr.it/ogis/ogis.php). The insulin early phase responses were captured by insulinogenic index (IGI_{30}) [76] and Stumvoll first (ISI_{first}), whilst the late phase by Stumvoll second phase (ISI_{second}) insulin sensitivity indices [74]. The pancreatic β-cell function was calculated using the Insulin Secretion-Sensitivity Index-2 (ISSI-2) [77] and oral disposition index (DI) calculated as ratio of IGI_{30} to fasting insulin [78]. The metabolic glucose clearance rate (MCR) was also calculated [74]. The aforementioned glucose and insulin indices have been selected based on their reproducibility as surrogate measures deriving from the OGTT procedure [46, 79-82]. Refer to **Appendix 4.1** for the formulas and calculations of indices used.

5.2.5 Analysis of blood samples

Plasma glucose and insulin samples were measured in a commercial laboratory (Waitemata District Health Board North Shore Hospital, Auckland, New Zealand). Plasma glucose concentrations were measured by the hexokinase method [83] (Vista GLU Flex® reagent cartridge, total CV 2-3%, Dimension Vista® 1500 System, Siemens Healthcare Limited). Plasma insulin was measured by a two-site sandwich immunoassay using direct chemiluminescent technology [84] (ADVIA Centaur Insulin assay, total CV 6.3-7.5%, Siemens ADVIA Centaur XP, Siemens Healthcare Limited).

5.2.6 Stratification into prediabetes subgroups

As impaired glycaemic control involves a set of heterogeneous disorders [55], it is useful in the present study to subdivide them into two distinct metabolic subgroups (healthier versus less healthy group) to determine their respective response to intervention using the Takahashi et al. (2018) stratification method [56]. Secondary analysis was therefore performed on subgroups of the prediabetes cohort. The healthier subgroup (n=10) consisted of individuals having both normal glucose and insulin peak times (30 min) or normal glucose peak time (30 min) with a delayed insulin peak (60 min). The less healthy subgroup (n=9) had delayed glucose (60 min) with delayed insulin peaks (≥60 min), or normal glucose peak time (30 min) with very delayed insulin peaks (120 min). Refer to **Appendix 4.2** for details of stratification methods considered.

5.2.7 Statistical analysis

Statistical analyses were performed using IBM SPSS statistics version 27 (IBM corporation, New York, US). A power calculation was performed based on Moore et. al. study, with an effect reduction of 100 mmol.min/L targeted and at least 16 participants were required [85, 86]. A linear mixed model for repeated-measures using the repeated covariance compound symmetry with estimation employing restricted maximum likelihood (REML) was employed. Bonferroni pairwise comparison was used to observe for potential differences between treatments when required. A p value of ≤ 0.05 was considered to be significant (95% confidence level). A p value of <0.10 was also considered as a trend tending towards statistical significance. The data are expressed as model-adjusted mean ± standard error of the mean (SEM).

A secondary analysis was performed on subgroups of the prediabetes cohort. Takahashi et al. (2018) stratification method [56] was used to subdivide the GLARE study

participants into two distinct metabolic subgroups (healthier versus less healthy group) to determine their respective responses to the intervention. Similarly, secondary analysis was conducted using linear mixed model for repeated-measures with repeated covariance compound symmetry after stratifying participants into two distinct metabolic profile subgroups (healthier versus less healthy group). A p value of ≤ 0.05 was considered to be significant (95% confidence level). A p value of <0.10 was also considered as a trend tending towards statistical significance. Differences between the two subgroups were analysed using unpaired independent Student t-test assuming equal variances. The data are expressed as model-adjusted mean ± standard error of the mean (SEM).

5.3 Results

5.3.1 Participant characteristics at baseline

Nineteen participants with prediabetes (five men and fourteen women) completed the study (Refer to **Figure 5.1** for CONSORT flow diagram under appendix of this chapter). The overall mean age of the participants was 65.0 ± 1.6 years of age, with a mean Body Mass Index (BMI) of 27.3 ± 1.1 kg/m^2, and mean HbA1c value of 42 ± 1 mmol/mol. Participant baseline characteristics are reported in **Table 5.1**. Eight out of 19 participants had other accompanying comorbidities such as hypertension, high cholesterol and heart disease and were taking medications on a consistent basis. These medications were assessed to have no to minimal influence on glucose metabolism. Two participants were withdrawn from the study due to problems obtaining enough blood samples via cannulation during visits.

Stratification of the participants into the two subgroups of prediabetes (healthier versus less healthy group) was based on their glucose and insulin peak times at baseline [56]. The baseline characteristics of the participants after the stratification are presented in **Table 5.1**. Baseline 1h and 2h postprandial glucose, as well as 2hPI were significantly different between the two groups ($p<0.05$), an indication of significant differences in glucose metabolic profiles.

Table 5.1 Baseline characteristics of participants in the GLARE study

Characteristics	Overall mean (± SEM)	Healthier subgroup mean (± SEM)	Less healthy subgroup mean (± SEM)	*p* value between healthier and less healthy subgroups
N	19	10	9	NA
Gender (M/F)	5/14	2/8	3/6	NA
Age (years)	65.0 ± 1.6	63.0 ± 2.5	67.2 ± 1.9	0.21
Body Mass Index (kg/m^2)	27.3 ± 1.1	26.0 ± 1.7	28.6 ± 1.3	0.25
Fasting blood glucose (mmol/L)	5.7 ± 0.1	5.6 ± 0.2	5.9 ± 0.2	0.37
1h postprandial glucose (mmol/L)	8.5 ± 0.7	6.6 ± 0.5	10.6 ± 0.9	0.001*
2h postprandial glucose (mmol/L)	6.9 ± 0.7	5.0 ± 0.3	8.9 ± 1.2	0.003*
Fasting insulin (mU/L)	8.0 ± 1.1	7.2 ± 1.2	9.0 ± 2.0	0.42
1h postprandial insulin (mU/L)	70.5 ± 9.2	78.4 ± 12.1	61.8 ± 14.2	0.39
2h postprandial insulin (mU/L)	50.5 ± 5.8	38.6 ± 6.5	70.1 ± 11.0	0.022*
Glycated haemoglobin A1c (mmol/mol)	42 ± 1	41 ± 1	43 ± 1	0.25
Systolic blood pressure (mm Hg)	135 ± 5	130 ± 5	141 ± 8	0.24
Diastolic blood pressure (mm Hg)	79 ± 3	79 ± 5	80 ± 4	0.92

Values were presented as means ± SEM. The less healthy subgroup consisted of participants with combined delayed glucose (60 min) and delayed insulin peaks (≥60 min), or normal glucose peak time (30 min) with very delayed insulin peaks (120 min). * indicates significant difference between healthier and less healthy subgroups, $p<0.05$ using the student t-test assuming equal variance.

5.3.2 Plant extracts and their impact on overall glucose and insulin indices in participants

The impact of each plant extract (grape seed, rooibos tea and olive leaf) on glucose and insulin responses in comparison to placebo are shown in **Table 5.2**. Overall, there were no significant differences in glucose and insulin indices between plant extracts and placebo, or between the plant extracts ($p>0.05$).

Table 5.2 Overall results of glycaemic indices of participants in all the trials

Analysis	Control (placebo)	Grape seed	Rooibos tea	Olive leaf	*p* value
Glucose indices					
Mean incremental area under the curve of glucose (mmol/L.min)	266.4 ± 46.3	256.5 ± 46.3	286.2 ± 46.8	284.2 ± 46.8	0.61
2h postprandial glucose (mmol/L)	6.9 ± 0.7	6.6 ± 0.7	6.9 ± 0.7	7.0 ± 0.7	0.82
Glucose peak time (min)	39.5 ± 4.1	43.4 ± 4.1	43.5 ± 4.2	36.8 ± 4.2	0.10
Metabolic clearance rate of glucose (mL/kg.min)	7.5 ± 0.6	7.6 ± 0.6	7.5 ± 0.6	7.1 ± 0.6	0.31
Insulin sensitivity indices					
Matsuda index	5.3 ± 0.7	5.3 ± 0.7	5.4 ± 0.7	5.1 ± 0.7	0.87
Oral glucose insulin sensitivity (mL/min.m^2)	380.9 ± 13.2	379.3 ± 13.2	385.9 ± 13.4	377.4 ± 13.4	0.80
Stumvoll overall insulin sensitivity index (pmol/L)	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.15
Early and late phase insulin response and sensitivity indices					
Insulinogenic index	0.9 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	0.63
Stumvoll first phase insulin sensitivity index (pmol/L)	1138.1 ± 144.8	1180.0 ± 144.8	1113.9 ± 146.7	1165.4 ± 146.7	0.87
Stumvoll second phase insulin sensitivity index (pmol/L)	304.7 ± 34.7	314.6 ± 34.7	299.4 ± 35.1	311.8 ± 35.1	0.88
β-cell function indices					
Insulin-secretion-sensitivity-index	34.0 ± 3.5	33.7 ± 3.5	33.7 ± 3.6	34.5 ± 3.6	1.00
Oral disposition index (by insulin)	0.12 ± 0.03	0.14 ± 0.03	0.13 ± 0.03	0.15 ± 0.03	0.68
Insulin secretion and response indices					
Mean incremental area under the curve of insulin (mU/L.min)	5663.3 ± 696.7	5794.7 ± 696.7	5714.6 ± 705.3	6300.4 ± 705.3	0.35
2h postprandial insulin (mU/L)	53.5 ± 7.1	50.9 ± 7.1	51.4 ± 7.3	62.4 ± 7.3	0.15
Insulin peak time (min)	75.8 ± 6.4	74.2 ± 6.4	82.9 ± 6.7	67.1 ± 6.7	0.15

Values were adjusted means ± SEM based on linear mixed model using compound symmetry as repeated covariance with restricted maximum likelihood (REML) estimation.

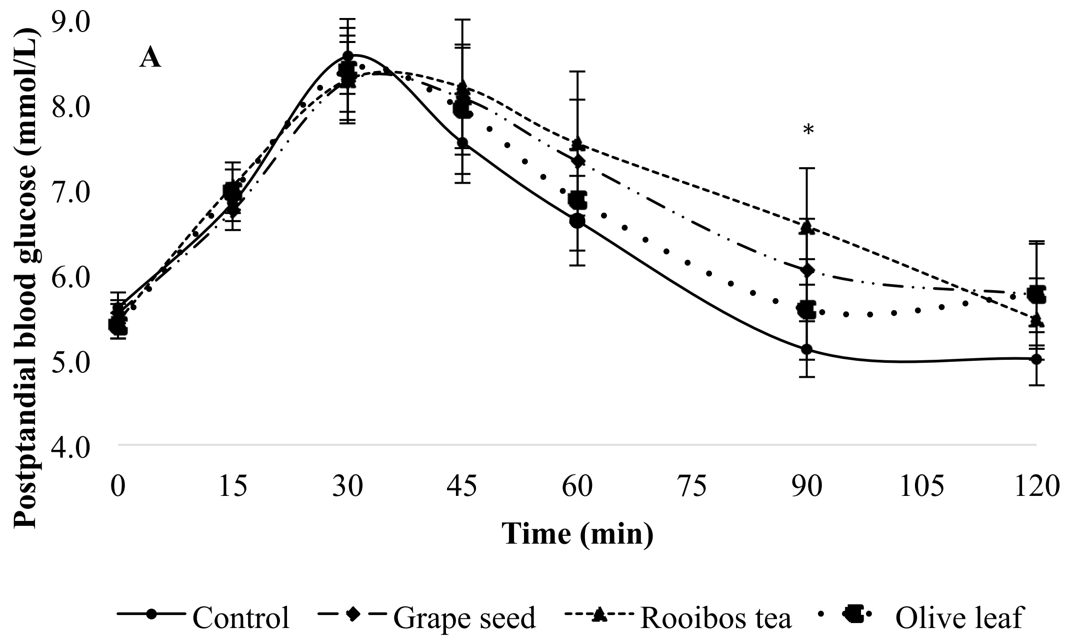
5.3.3 Plant extracts and their impact on glucose and insulin indices after stratification of participants based on glucose and insulin peak time patterns

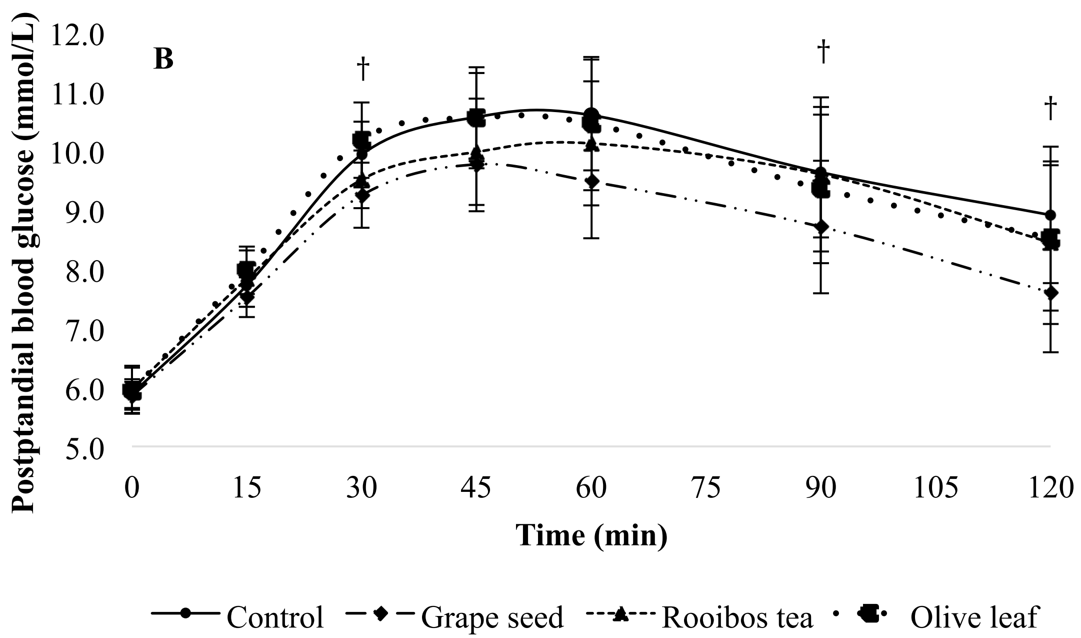
Figure 5.2 shows the postprandial glucose and insulin responses of the participants in the healthier and less healthy subgroups after the stratification based on the Takahashi et al. method (2018).

In the healthier subgroup, there was no significant impact on either glucose or insulin indices with any of the three plant extracts except for a significantly higher 2hPI in the olive leaf extract treatment compared to placebo (p=0.030, 49.5% higher) (**Table 5.3**). The higher insulin concentration inevitably led to a reduced $ISI_{overall}$ (p=0.032, 12.5% decrease) and MCR (p=0.040, 10.2% decrease).

In the less healthy subgroup, grape seed was shown to significantly reduce $iAUC_{glucose}$ (p=0.016, 21.9% reduction), 2hPG (p=0.034, 14.7% reduction), 2hPI (p=0.029, 22.4% reduction), and improve $ISI_{overall}$ (p=0.028, 15.0% increase) and MCR (p=0.016, 16.7% increase) compared to placebo (**Table 5.3**). Rooibos tea extract significantly improved DI (p=0.031, 32.4% increase) and showed trend toward improvement for ISSI-2 (p=0.07, 18.3% increase) compared to placebo (**Table 5.3**). Olive leaf extract was observed to significantly increase $iAUC_{insulin}$ (p=0.040, 16.7% increase), and showed trend toward improvement for ISI_{first} (p=0.08, 17.8% increase), $ISI_{second,}$ (p=0.06, 15.6% increase), as well as IGI_{30} (p=0.08, 27.8% increase) compared to placebo (**Table 5.3**).

There were no significant differences in both glucose and insulin indices amongst the treatments in both subgroups (p>0.05).


A
Postptandial blood glucose (mmol/L)
9.0
8.0
7.0
6.0
5.0
4.0
*
0
15
30
45
60
75
90
105
120
Time (min)
Control
Grape seed
Rooibos tea
Olive leaf


B
Postptandial blood glucose (mmol/L)
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
†
†
†
0
15
30
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60
75
90
105
120
Time (min)
Control
Grape seed
Rooibos tea
Olive leaf

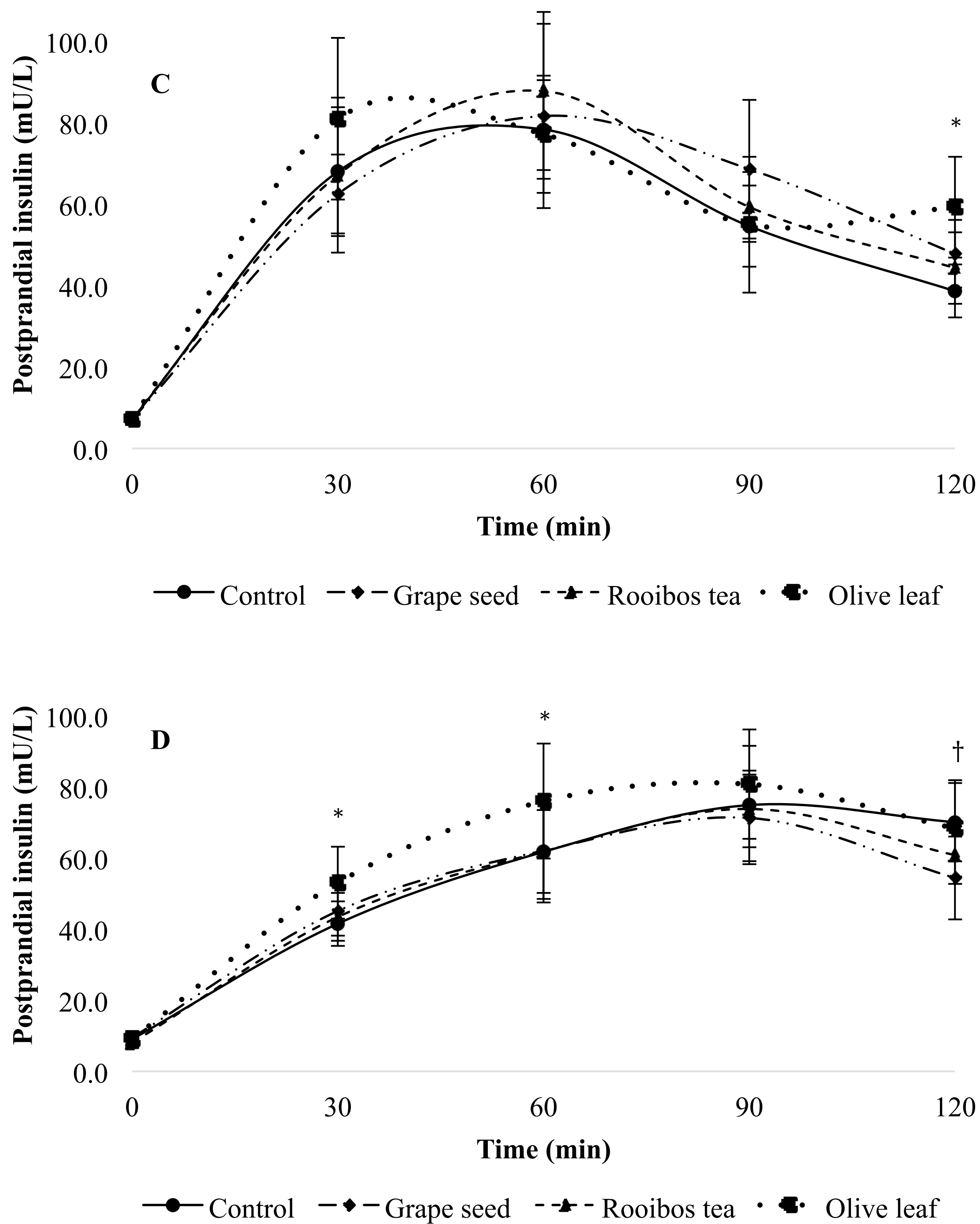

Figure 5.2 Unadjusted mean postprandial plasma glucose and insulin of participants during an oral glucose tolerance test (OGTT). (A) **Postprandial glucose of participants in the healthier subgroup (n=10) (normal glucose and insulin peak times (30 min) or delayed insulin peak (60 min) at control visit and treatment visits.** (B) **Postprandial glucose of participants in the less healthy subgroup (n=9) (combined delayed glucose (60 min) and delayed insulin peaks (≥60 min), or normal glucose peak time (30 min) with very delayed insulin peaks (120 min)) at control visit and treatment visits.** (C) **Postprandial insulin of participants in the healthier subgroup (n=10).** (D) **Postprandial insulin of participants in the less healthy subgroup (n=9). Linear mixed model for repeated-measures with repeated covariance compound symmetry was used to derive statistical significance.** * **indicates treatment with extract was significantly higher than placebo (*p*<0.05).** † **indicates treatment with extract was significantly lower than placebo (*p*<0.05).**

Table 5.3 Results of glycaemic indices of participants with prediabetes in the healthier and less healthy subgroups

Analysis	Healthier subgroup					Less healthy subgroup				
	Control (placebo)	Grape seed	Rooibos tea	Olive leaf	*p* value	Control (placebo)	Grape seed	Rooibos tea	Olive leaf	*p* value
Glucose indices										
Mean incremental area under the curve of glucose (mmol/L.min)	126.3 ± 38.5	190.8 ± 38.5*	203.1 ± 40.1*	171.0 ± 40.1	0.050	422.0 ± 71.4	329.5 ± 71.4*	381.0 ± 71.4	405.7 ± 71.4	0.08
2h postprandial glucose (mmol/L)	5.0 ± 0.5	5.8 ± 0.5	5.4 ± 0.5	5.7 ± 0.5	0.51	8.9 ± 1.2	7.6 ± 1.2*	8.4 ± 1.2	8.5 ± 1.2	0.18
Glucose peak time (min)	31.5 ± 3.2	39.0 ± 3.2	34.2 ± 3.5	31.2 ± 3.5	0.16	48.3 ± 7.3	48.3 ± 7.3	53.3 ± 7.3	43.3 ± 7.3	0.23
Metabolic clearance rate of glucose (mL/kg.min)	8.9 ± 0.7	8.4 ± 0.7	8.6 ± 0.7	8.0 ± 0.7*	0.18	5.8 ± 0.8	6.8 ± 0.8*	6.3 ± 0.8	6.0 ± 0.8	0.08
Insulin sensitivity indices										
Matsuda index	5.9 ± 1.0	5.7 ± 1.0	5.9 ± 1.0	5.9 ± 1.0	0.99	4.6 ± 0.8	4.7 ± 0.8	4.8 ± 0.8	4.2 ± 0.8	0.26
Oral glucose insulin sensitivity ($mL/min.m^2$)	405.4 ± 16.8	392.2 ± 16.8	410.9 ± 17.7	402.3 ± 17.7	0.62	353.7 ± 18.3	365.0 ± 18.3	360.0 ± 18.3	351.6 ± 18.3	0.53
Stumvoll overall insulin sensitivity index (pmol/L)	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01*	0.15	0.06 ± 0.01	0.07 ± 0.01*	0.07 ± 0.01	0.06 ± 0.01	0.10
Early and late phase insulin response and sensitivity indices										
Insulinogenic index	1.3 ± 0.4	1.4 ± 0.4	1.2 ± 0.4	1.5 ± 0.4	0.84	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.24
Stumvoll first phase insulin sensitivity index (pmol/L)	1389.8 ± 209.9	1335.3 ± 209.9	1321.5 ± 218.0	1295.2 ± 218.0	0.93	858.4 ± 185.4	1007.5 ± 185.4	890.3 ± 185.4	1011.1 ± 185.4	0.17
Stumvoll second phase insulin sensitivity index (pmol/L)	362.2 ± 50.3	351.2 ± 50.3	348.6 ± 52.4	339.5 ± 52.4	0.94	240.8 ± 44.9	273.9 ± 44.9	246.9 ± 44.9	278.4 ± 44.9	0.15
β-cell function indices										
Insulin-secretion-sensitivity-index	43.7 ± 4.4	41.5 ± 4.4	38.9 ± 4.6	42.6 ± 4.6	0.58	23.2 ± 4.2	25.0 ± 4.2	27.4 ± 4.2	25.6 ± 4.2	0.34
Oral disposition index (by insulin)	0.18 ± 0.04	0.20 ± 0.04	0.16 ± 0.05	0.22 ± 0.05	0.65	0.07 ± 0.02	0.08 ± 0.02	0.09 ± 0.02*	0.08 ± 0.02	0.18
Insulin secretion and response indices										
Mean incremental area under the curve of insulin (mU/L.min)	5857.1 ± 991.3	6352.2 ± 991.3	5935.7 ± 1024.6	6158.6 ± 1024.6	0.85	5448.0 ± 1031.8	5175.2 ± 1031.8	5451.0 ± 1031.8	6359.3 ± 1031.8*	0.048
2h postprandial insulin (mU/L)	38.6 ± 8.4	47.8 ± 8.4	42.8 ± 9.0	57.8 ± 9.0*	0.15	70.1 ± 11.3	54.4 ± 11.3*	60.8 ± 11.3	68.4 ± 11.3	0.11
Insulin peak time (min)	57.0 ± 7.5	69.0 ± 7.5	64.7 ± 8.3	49.7 ± 8.3	0.27	96.7 ± 7.7	80.0 ± 7.7	100.0 ± 7.7	83.3 ± 7.7	0.06

Values were adjusted means ± SEM based on linear mixed model using compound symmetry as repeated covariance with restricted maximum likelihood (REML) estimation. * indicates significant difference ($p<0.05$) between treatment and placebo.

5.4 Discussion

Plant extracts have been increasingly gaining attention over the past decade as functional agents to improve glycaemic control [10, 87, 88]. The uniqueness of the plant extracts used in the present study was the high concentrations of polyphenols and antioxidants they contained that could potentially have an impact on glucose metabolism [27-29, 32, 33, 35, 89-93].

To the best of our knowledge the present study is the first to investigate the hypoglycaemic impact of grape seed, rooibos tea, and olive leaf extracts in a solely prediabetes cohort. No significant results were seen for the group as a whole. As impaired glycaemic control involves a set of heterogeneous disorders [55], we stratified the GLARE study participants into two distinct metabolic subgroups (healthier versus less healthy group) [56] and observed significant differences in response to the intervention given.

At baseline, participants in the healthier subgroup had significantly lower glucose and glucose peak time values, lower 2hPI and insulin peak time, higher OGIS insulin sensitivity, Stumvoll $ISI_{overall}$, ISI_{first}, ISI_{second} and IGI_{30}, higher β-cell function and MCR compared to the less healthy subgroup. The differences in glucose and insulin levels as well as insulin sensitivity justify the stratification of participants into two subgroups with distinct glucose metabolic profiles.

There were no significant changes in glucose and insulin responses in the healthier subgroup compared to placebo nor between treatments ($p>0.05$), except for an increased 2hPI and reduced insulin sensitivity in the olive leaf extract due to the increased insulin level compared to placebo. Although there was a significantly greater $iAUC_{glucose}$ with grape seed ($p=0.022$) and rooibos tea ($p=0.013$) extracts compared to placebo in the healthier subgroup, the values were significantly lower than the baseline values of the less healthy subgroup.

In the less healthy subgroup, grape seed extract was shown to be the most effective in improving glucose and insulin responses compared to rooibos tea or olive leaf extract. Postprandial glucose and insulin concentrations were significantly reduced ($p<0.05$), whilst there was significant improvement in overall insulin sensitivity ($p=0.028$) and MCR ($p=0.016$) compared to placebo. This finding agrees with previous acute randomised controlled trials investigating grape seed extracts of various concentrations (100-500 mg) and they were found to improve markers of glucose metabolism in healthy individuals and those with metabolic syndrome [23, 27, 29]. However the findings were in contrast to other chronic

trials on healthy participants and those with T2DM or metabolic syndrome that showed no significant improvement in glycaemia with grape seed extract (150-400 mg/day) [21, 22, 24, 26]. Differences in the extract grade, doses, small sample sizes and study duration might explain the inconsistencies in results.

Rooibos tea extract was shown to improve the DI (p=0.031, 32.4% increase) indicating the possibility of potential improvement to the β-cell function in the less healthy subgroup. This was coupled with improved insulin sensitivity ISSI-2 (p=0.07, 18.3% increase). These improvements were indicative of improved glycaemic control and were aligned with research showing that restoring β-cell function is pivotal to restoring glucose homeostasis and delaying T2DM development [94-97]. A previous clinical trial showed significant reduction in $iAUC_{glucose}$ in healthy participants after consuming 760 mg of rooibos tea extract [29]. However, in the present study although the $iAUC_{glucose}$ (p=0.26, 9.7% reduction) and 2hPG (p=0.43, 5.2% reduction) were reduced with an increase in MCR (p=0.20, 8.4% increase) indicating higher glucose clearance with rooibos tea extract compared to placebo, these did not reach statistical significance. The difference in results might be due to the metabolic differences in the participants in the two studies and the higher dose used in the previous study.

Olive leaf extract was consistently shown to elevate insulin levels in the present study, with a higher 2hPI in the healthier subgroup (p=0.030) and an elevated $iAUC_{insulin}$ in the less healthy (p=0.040) subgroups compared to placebo. Prior research has demonstrated that olive leaf might be an insulin secretagogue and might be suitable for hyperglycaemic individuals secreting low levels of insulin due to impairment of the pancreas [98, 99]. However, clinical trials have shown a decrease in insulin secretion with subsequent improvement in insulin sensitivity. de Bock and co-workers (2013) conducted a 12-week clinical study on olive leaf extract (51.1 mg oleuropein, 9.7 mg hydroxytyrosol/day) and demonstrated a reduction in insulin secretion with subsequent improvement in insulin sensitivity and postprandial glycaemia in overweight participants [33]. Similarly, Wainstein and colleagues (2012) also demonstrated significant reduction in fasting insulin with accompanying improvement in glucose response in T2DM participants after consuming 500 mg/day of olive leaf extract for 14 weeks [32]. Komaki and colleagues (2003) also demonstrated that 1000 mg of olive leaves consumed with 300 g of cooked rice in a 3h OGTT significantly reduced PG at 30 min and 1h in borderline diabetic subgroup (n=7; FBG of 6.1-7.8 mmol/L) [31]. Araki and colleagues (2019) also demonstrated significant reduction in FBG in participants with prediabetes after a 12-week olive leaf tea consumption (32.4

mg/100 g oleuropein, 1.2 mg/100 g hydroxytyrosol in 330 mL of tea beverage three times a day) [37].

In contrast, a six-week chronic study showed no significant improvements in fasting glucose or insulin sensitivity with the consumption of a supplement mix containing 500 mg/day of olive leaf (80-120 mg oleuropein/day) along with other extracts in hypertensive men [34]. Likewise, no significant improvements in glucose and insulin responses were seen with pre-hypertensive men after consuming olive leaf extract in juice form (136 mg oleuropein, 6 mg hydroxytyrosol/day) for six weeks [35]. The extract types and doses, study duration and metabolic profile of participants might have contributed to the differences in results observed in comparison to the present acute study.

A delay in insulin peak or a loss of early phase insulin response is often a glucose metabolic defect associated with impaired glycaemic control [100, 101]. Early phase insulin response is critical for maintaining glucose homeostasis in the postprandial state by suppressing glucagon secretion and inhibiting hepatic glucose production [102-104]. Studies have revealed the possibility of restoring healthy postprandial glucose levels or reversing β-cell function by regenerating the first phase insulin response [103-105]. The present study showed that grape seed was able to shorten the time to insulin peak in the less healthy subgroup (p=0.054). This was accompanied by a non-significant improvement in ISI_{first} (p=0.08, 17.4% increase) and IGI_{30} (p=0.08, 27.8% increase). Olive leaf extract was also shown to improve both ISI_{first} (p=0.08, 17.8% increase) and IGI_{30} (p=0.08, 27.8% increase) in the less healthy subgroup. Similarly in the study conducted by de Bock and colleagues (2013), IGI_{30} was significantly improved (p=0.013) in non-diabetic, overweight men after a 12-week consumption of olive leaf extract [33]. Therefore, although changes were not significant, this could indicate a possibility of restoring an earlier insulin secretion and sensitivity with grape seed and olive leaf extracts. Research has shown that the second or late phase insulin secretion is also critically important and is an independent predictor of T2DM [106, 107]. In the less healthy subgroup, second phase insulin sensitivity ISI_{second} was improved with grape seed (p=0.10, 13.7% increase), as well as with olive leaf (p=0.06, 15.6% increase), however not reaching statistical significance.

Although the plant extracts tested were matched for total antioxidant capacity to efficacy comparison, it was observed that the outcomes were not similar amongst the extracts. Several studies have corroborated that the phenolic or antioxidant content of plant extracts might not necessarily correlate with the hypoglycaemic actions exhibited [108-111]. Rather, the presence of different phenolic compositions in the extracts work collectively to

structurally interact to inhibit digestive enzymes and glucose transporters including utilising other glucose metabolism pathways to impact glucose uptake and absorption [10, 11, 18, 19].

The strengths of the GLARE study included the use of a crossover design where participants were their own control resulting in a smaller sample size required. The plant extracts examined were also matched for TAC using the ORAC assay that is a well-researched method, and allowed the comparison of efficacy amongst the extracts.

Nevertheless, the present study is not without limitations. The current definition of prediabetes is somewhat arbitrary and is still expanding, and hence it is yet unclear whether it is truly a continuum or spectrum of heterogeneous worsening of glycaemic control [112], or whether it is defined by distinct metabolic phenotypes [45]. Studies have elucidated the existence of prediabetes spectrum: IFG, IGT and IFG/IGT, and demonstrated them to be driven by different underlying dysfunctional metabolic profiles of glucose metabolism that included differences in glucose and insulin patterns [100, 101]. The present study had recruited participants with prediabetes based solely on their HbA1c values and not on these distinct metabolic phenotypes as a much larger sample size would be required. Moreover, HbA1c measurement was also not able to distinguish between IFG and IGT individuals [113], resulting in a heterogeneous mixture of participants with various glycaemic profiles, as demonstrated by studies showing HbA1c-defined prediabetes as exhibiting a mixture of metabolic defects of both IFG and IGT [114-116]. In this study three participants had IFG/IGT, and two had IFG, and one had IGT in addition to having an elevated HbA1c value. The other participants exhibited normal fasting blood glucose (FBG) and 2hPG values but also had elevated HbA1c. For this present study stratification and investigation of the effectiveness of plant extracts on two metabolically distinct prediabetes subgroups based on glucose and insulin response patterns has occurred. However, a limitation of the stratification was the smaller sample size in each subgroup.

One of the merits of using HbA1c for prediabetes screening is its consistency in identifying individuals with prediabetes and its reproducibility [64, 115, 117, 118]. It is not uncommon that the day-to-day intra-individual variability in both FBG and 2hPG were greater than HbA1c [118-120], especially as individuals in transition from normoglycaemia into impaired glycaemic control tend to have higher variability in response to an OGTT to obtain glucose responses [121-125]. This was corroborated by a study that variation with 2hPG was 16.7% and FBG, 5.7%, and comparatively less reproducible to HbA1c with a variation of only 3.6% [119]. This might be due to HbA1c measuring chronic exposure to both basal and postprandial hyperglycaemia in contrast to FBG and 2hPG measurements

[126]. However, HbA1c values can be influenced by other factors such as ethnicity, age, gender, FPG, BMI, lifestyle and habits such as smoking and alcohol consumption, and haemoglobin related conditions [127-129]. This demonstrates the need to include a range of surrogate markers of glucose in order to refine the definition of prediabetes for a more accurate diagnosis [130, 131].

The GLARE study consisted of participants who were only having borderline prediabetes (mean HbA1c 42 ± 1), and study outcomes observed in this study might not be replicable to those with worsening glycaemic control and having a higher HbA1c reading closer to the diabetic range. Furthermore, some study participants (n=6), who at the point of screening, had HbA1c values (38-40 mmol/mol) below the NZSSD stipulated 41-49 mmol/mol [64]. However, the participants were recruited only after being assessed to have at least one risk factor for T2DM (overweight or obese, high blood pressure, had prediabetes before, and family history of T2DM or CVD). A study conducted by Marini et al. (2014) on 338 non-diabetic offspring of T2DM parents using American Diabetes Association criteria for prediabetes revealed that those with HbA1c values above 39 mmol/mol (5.7%) already suffered from a significant faltering insulin sensitivity and β-cell function [132]. Edelman et al. (2004) in their research also recommended closer scrutiny for patients with prediabetes having high-normal HbA1c (37.7-42.1 mmol/mol, 5.6-6.0%) to elevated HbA1c (43.2-51.9 mmol/mol, 6.1-6.9%) especially if they were obese, as their diabetes incidence were higher each year, 2.5% and 7.8%, respectively [133]. In addition, considering that red blood cell renewal cycle varies even in healthy individuals from 38-60 days and 39-56 days in diabetics, HbA1c readings might be significantly altered if measurements were taken at different times of the cycle [134]. Hence, these participants were included as part of the present study.

Future studies could also look at using mixed meals instead of liquid glucose as a better representation of a typical meal consumed [135]. A recent review concluded from studies investigating polyphenol-rich sources that glucose and insulin responses may differ based on the polyphenol-carbohydrate combination [136]. In contrast, another study discussed the merits of liquid glucose as opposed to mixed meals and concluded that glucose, insulin, C-peptide and β-cell function levels were comparable between standardised mixed meals and liquid glucose in both healthy and T2DM participants, although such responses were higher in healthy participants [137].

The present study has only collected data of glycaemic response based on a two-hour OGTT. Due to the high phenolic content present in the plant extracts, these plant extracts undergo extensive metabolism in the human body [138, 139]. This suggests that the potential

hypoglycaemic effects from the plant extracts may only become apparent after a prolonged period of metabolism. Future studies may investigate the metabolites generated from the consumption of the plant extracts to understand the type and extent of metabolism of the extracts in relation to their effects on glycaemic control.

Finally, it is recommended for future studies to measure C-peptide, which is co-secreted in equimolar amounts with insulin and does not undergo hepatic first pass before circulation and is not affected by impaired insulin clearance in insulin resistant individuals and hence represents a more accurate indication of insulin secretion [140-142]. Furthermore, C-peptide based indices have been shown to provide a better prediction and evaluation of progression to diabetes and therefore useful for the study of prediabetes [140, 143, 144].

In conclusion, the current study has shown the potential acute hypoglycaemic effects of grape seed, rooibos tea and olive leaf extracts in improving indices of glucose and insulin responses in individuals with prediabetes, particularly those with less healthy metabolic profiles. Future chronic study of the plant extracts in individuals with prediabetes will help to elucidate if their impact on glycaemic control and insulin sensitivity are sustainable.

Appendix

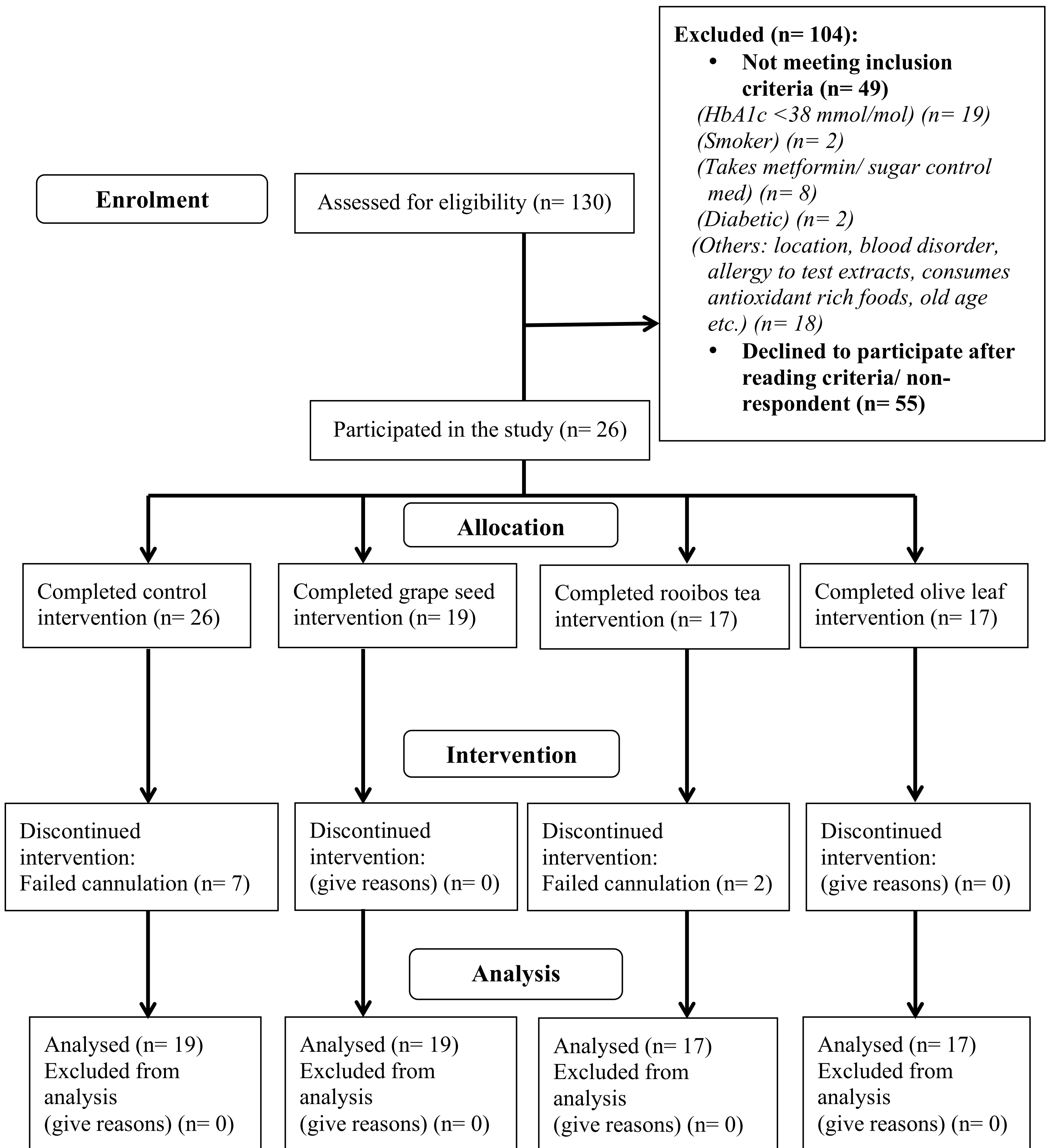

Figure 5.1 CONSORT flow diagram for the GLARE study

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Chapter 6

Short communication: Inhibitory actions of antioxidant-rich plant extracts on alpha-amylase and dipeptidyl peptidase-4 enzymatic activities. A mechanistic insight into their hypoglycaemic effects

This chapter presents the study outcomes examining potential mechanisms for the hypoglycaemic effects of the four plant extracts: grape seed, rooibos tea, olive leaf and New Zealand pine bark. The mechanistic study involved investigating the potential inhibition on enzymes involved in glycaemic control: α-amylase and dipeptidyl-peptidase-4. This chapter has been presented in a short-communication manuscript format and prepared for submission to the *Nutrients* Journal.

Abstract

Grape seed, rooibos tea, olive leaf and the New Zealand pine bark extracts have shown acute hypoglycaemic effects in humans. Assays of inhibition on digestive enzyme α-amylase and dipeptidyl-peptidase-4 enzyme as potential underlying mechanisms for improved glycaemic control were conducted on the extracts at various concentrations. Grape seed, rooibos tea and pine bark at 5 mg/mL inhibited α-amylase at 27.8% ± 1.0%, 32.7% ± 0.8%, and 58.9% ± 1.6%, respectively, with pine bark having the strongest inhibition ($p<0.001$). Grape seed, rooibos tea and pine bark at 5 mg/mL also inhibited dipeptidyl-peptidase-4 enzyme at 49.6% ± 0.4%, 57.2% ± 2.1% and 70.5% ± 1.1%, respectively. The IC_{50} of pine bark on α-amylase and dipeptidyl-peptidase-4 enzymes were 3.98 ± 0.11 mg/mL and 2.51 ± 0.04 mg/mL, respectively. Olive leaf extract did not inhibit any enzymes. The present findings indicate the potential for these extracts to improve postprandial glycaemia by delaying carbohydrate digestion and enhancing the incretin effect via the inhibition of digestive enzyme α-amylase and dipeptidyl-peptidase-4 enzyme, respectively.

Keywords: functional food; grape seed; rooibos tea; olive leaf; New Zealand pine bark; hyperglycaemia; impaired glycaemic control; hypoglycaemic effects

6.1 Introduction

There is increasing evidence that antioxidant-rich plant-derived extracts have properties that improve glucose metabolism and glycaemic control [1-3]. Inhibiting glucose-regulating enzymes such as α-amylase [4-8] and dipeptidyl peptidase-4 (DPP4) [9, 10] have been proposed as possible underlying mechanisms by which the plant extracts exert their hypoglycaemic effects.

Alpha-amylase is a key enzyme involved in the digestion of starch to glucose for absorption and therefore plays a crucial role in regulating postprandial glycaemia [2]. Salivary and pancreatic α-amylases catalyse the endo-hydrolysis of α-1,4-glucosidic linkages from starch amylose to release maltose, maltotriose and related α-1,6-oligomers that are eventually broken down by α-glucosidase to produce glucose [2]. Inhibiting α-amylase may prevent or slow carbohydrate digestion leading to subsequent suppression of postprandial hyperglycaemia, and therefore a useful mechanism for improving glycaemic control [4-8].

Dipeptidyl peptidase-4 enzyme plays a critical role in cleaving endogenous active forms of incretins such as gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) into inactive compounds leading to the loss of incretin effect [11-13]. The incretin effect is stimulated during a glucose load and is crucial for establishing postprandial glucose homeostasis by secreting insulin [14], suppressing glucagon release [15, 16] and hunger [17-19], and delaying gastric emptying [18, 20]. However, incretin levels or activity tend to decrease in situations of persistent hyperglycaemia such as in prediabetes and type 2 diabetes mellitus (T2DM) [13, 21-29]. Inhibiting DPP4 enzyme may help preserve circulating endogenous incretins and their activity prolonged in the body to regulate postprandial glycaemia [9, 10, 30, 31].

We have previously demonstrated that the New Zealand pine bark exhibited the ability to moderate glycaemic control in healthy participants (Pine Bark study) [32], whilst grape seed, rooibos tea and olive leaf extracts showed significant impact on glycaemic indices in participants with prediabetes in the GLARE study (unpublished). However, the specific mechanisms of hypoglycaemic action of these extracts have yet to be fully elucidated. Several *in vitro* and *in vivo* studies have investigated the inhibitory effects of grape seed [33-37], rooibos tea [38, 39], olive leaf [36, 40-47], and pine bark [48-50] extracts on α-amylase enzyme though comparative results are inconclusive due to differences in test products and methodologies [36, 51]. The inhibition of DPP4 enzyme has not been explored with rooibos tea, olive leaf, and pine bark extracts. Only grape seed extract has been studied

to show inhibitory action on DPP4 enzyme [52, 53]. Thus, the aim of the current study was to investigate the potential inhibitory action of grape seed, rooibos tea, olive leaf and New Zealand pine bark extracts on two key digestive enzymes important for glucose metabolism: α-amylase and DPP4. We have also determined the IC_{50} values (concentration of extract required to inhibit 50% of enzymatic activity) of the New Zealand pine bark extract on α-amylase and DPP4 enzymes as this has not been previously reported.

6.2 Methods

6.2.1 Materials

Grape seed extract (*Vitis vinifera*) (Nutra-Life, Vitaco Health (NZ) Limited), unfermented green rooibos tea extract (*Aspalathus linearis,* E2CCJ) (Rooibos Limited, South Africa), olive leaf extract (*Olea europaea*) (Comvita NZ Limited), and the New Zealand pine bark extract (*Pinus radiata*) (ENZO nutraceutical Limited) were obtained commercially.

All chemicals used for the analysis and sample preparation were of analytical grade. Ultrapure water was prepared using a Millipore water purification system. Stock phosphate saline buffer (PBS, pH 6.8-6.9) was purchased from Thermo Fisher Scientific. Acarbose, α-amylase from porcine pancreas, 3,5-dinitrosalicylic acid (DNS) reagent, and soluble starch (potato), were purchased from Sigma-Aldrich. The DPP4 inhibitor screening assay kit was purchased from Abcam (Ab133081, Cambridge, UK). The kit contained human recombinant DPP4 enzyme, dipeptidyl-peptidase (DPP) substrate (H-Gly-Pro-AMC), assay buffer (20mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1 mM EDTA), and sitagliptin as a positive control inhibitor.

6.2.2 Sample preparation

Sample extracts of grape seed, rooibos tea, olive leaf and New Zealand pine bark were sent to Callaghan Innovation (Wellington, New Zealand) to determine their inhibition on α-amylase and DPP4 enzymes. Powdered extracts of grape seed, rooibos tea and pine bark were solubilised in dimethyl sulfoxide (DMSO) at 20 mg/mL and diluted with pure water (Milli-Q) into various concentrations. The olive leaf extract was dissolved in a solvent mixture (ethanol:DMSO 4:1) to 40 mg/mL as a stock solution, then diluted with Milli-Q water into various concentrations selected based on a previous study [40].

6.2.3 Enzyme inhibition assays

α-amylase and DPP4 enzyme inhibition assays were conducted on extracts of grape seed, rooibos tea, olive leaf and pine bark based on previous studies [40, 54] with some modifications. At least two concentrations of each plant extract were tested. In cases where negligible inhibition was observed, higher concentrations were tested. All samples were performed in 96-well plate format on a microreader (SpectraMax 4M, Molecular Devices). The positive controls acarbose and sitagliptin were tested at two concentrations at least to ensure assays were working correctly.

6.2.3.1 α-amylase inhibition assay

Grape seed and rooibos tea extracts were measured at 2 and 5 mg/mL. Olive leaf extract was measured at 2, 5, 10 and 20 mg/mL. In order to determine the IC_{50} value, pine bark extract was measured at 0.5, 1, 2.5, 5 and 10 mg/mL. Positive control inhibitor acarbose was measured at 2 and 5 mg/mL. Samples were incubated with α-amylase at 30°C for 15 min before the addition of 1% starch solution. The hydrolysis of starch by α-amylase in the absence and presence of the sample was kept at 30°C for 30 min and stopped by adding 1% DNS solution. The mixture was heated at 100°C for 10 min and then diluted 4-fold with water before being read at 540 nm.

6.2.3.2 Dipeptidyl peptidase-4 (DPP4) inhibition assay

Grape seed and rooibos tea extracts were measured at 1 and 5 mg/mL. Olive leaf extract was measured at 1, 5, 10 and 20 mg/mL. In order to determine the IC_{50} value, pine bark extract was measured at 0.25, 0.5, 1, 2.5 and 5 mg/mL. Positive control inhibitor sitagliptin was measured at 0.001, 0.01, 0.1, 1, and 10 M. The reaction was initiated by the addition of DPP substrate and incubated at 37°C for 30 min. Readings were done at Ex355 nm and Em 460 nm.

6.2.4 IC_{50} determination of pine bark extract on enzymes

The IC_{50} values of the pine bark extract on α-amylase and DPP4 enzyme inhibition were determined using similar methodology as a previous study [40]. The IC_{50} was defined as the concentration of an inhibitor required for reducing 50% of the enzyme activity obtained from a dose-dependent activity versus concentration plot. The data points of five different concentrations of pine bark extract were fitted into a non-linear sigmoid plot to take into account non-linear concentration dependent of enzyme-inhibitor interaction at low and high concentrations.

6.2.5 Statistical analysis

Statistical analysis was performed by general linear model univariate analysis using SPSS software version 25 (IBM Corporation, New York, NY, USA) with Tukey-Kramer multiple comparison test ($p \leq 0.05$) to compare between plant extracts. The data are presented as mean ± SEM, with a minimum of $n=3$ samples of the same batch tested.

6.3 Results

6.3.1 α-amylase activity inhibition

At a concentration of 5 mg/mL, pine bark extract showed the highest α-amylase inhibition (58.9% ± 1.6%), followed by rooibos tea (32.7% ± 0.8%), then grape seed (27.8% ± 1.0%) (**Figure 6.1**). There was a significant difference between pine bark and rooibos tea ($p<0.001$), as well as pine bark and grape seed ($p<0.001$). However, there was no significant difference between grape seed and rooibos tea ($p=0.066$). Pine bark, grape seed and rooibos tea extracts had lower inhibitory activity than acarbose (74.3% ± 6.3%) at a similar concentration, however the difference was statistically insignificant ($p=0.241$). Olive leaf extract had negligible inhibition against α-amylase at similar concentrations, but showed inhibition of 29.2% ± 2.7% only at a higher concentration of 20 mg/mL.

6.3.2 Dipeptidyl peptidase-4 (DPP4) activity inhibition

At a concentration of 5mg/mL, pine bark extract showed the highest DPP4 inhibition (70.5% ± 1.1%), followed by rooibos tea (57.2% ± 2.1%), then grape seed (49.6% ± 0.4%) (**Figure 6.1**). The DPP4 inhibition with pine bark was significantly stronger than grape seed ($p<0.001$) and rooibos tea ($p=0.001$). Rooibos tea was able to inhibit DPP4 enzyme to a greater extent than grape seed ($p=0.018$). Their inhibitory activities were lower compared to that of sitagliptin (97.4% at 4.1×10^{-4} mg/mL). Olive leaf extract had negligible inhibition against DPP4 enzyme at all concentrations tested.

6.3.3 IC_{50} of pine bark extract on enzymes

The IC_{50} values of pine bark extract for α-amylase and DPP4 enzymes were 3.98 ± 0.11 mg/mL and 2.51 ± 0.04 mg/mL, respectively.

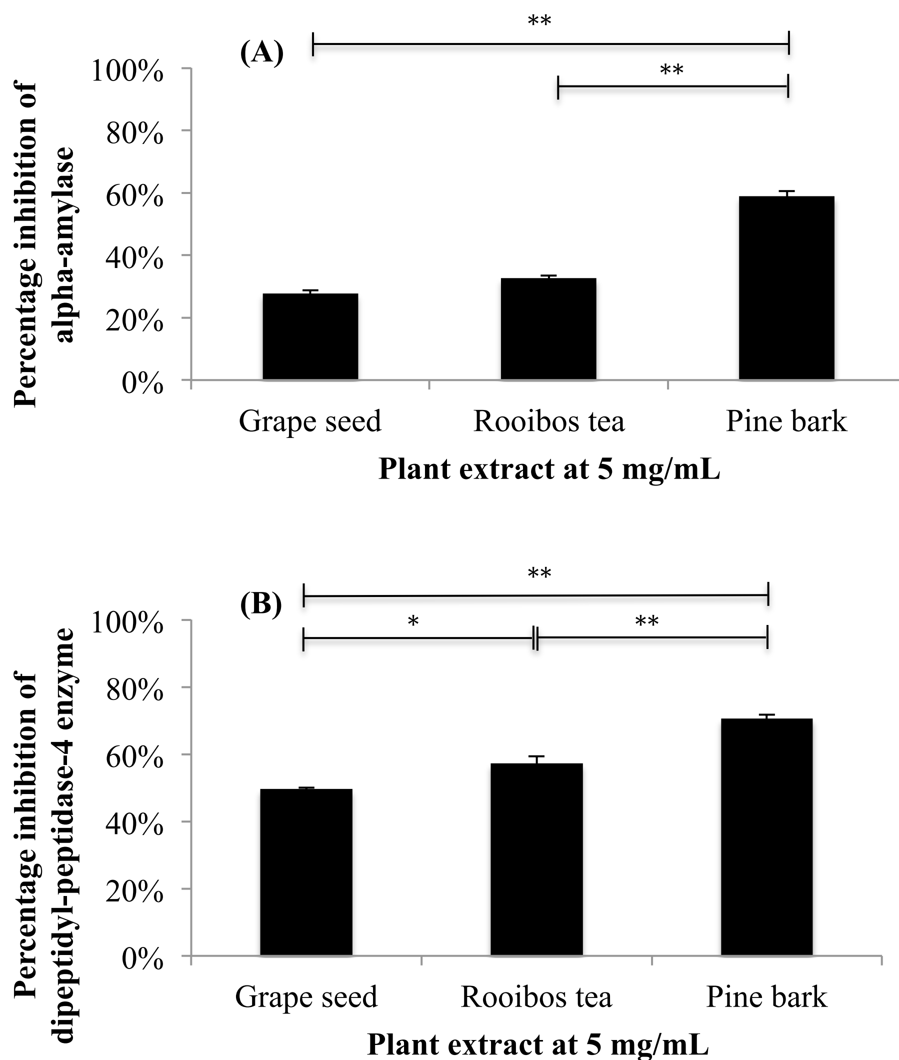

Figure 6.1 Bar charts showing percentage inhibition of grape seed, rooibos tea and pine bark extracts on (A) α-amylase at 5 mg/mL and (B) dipeptidyl-peptidase-4 (DPP4) enzyme at 5 mg/mL. Data are presented as mean percentage inhibition ± SEM. * indicates significant difference of $p<0.05$. ** indicates significant difference of $p\leq0.001$.

6.4 Discussion

The present study demonstrated that the New Zealand pine bark extract exhibited the greatest inhibitory effects against digestive enzyme α-amylase and DPP4 enzyme for the four extracts. Grape seed and rooibos tea extracts showed good inhibition of both enzymes tested. Whereas, olive leaf extract showed minimal inhibition on α-amylase and no inhibition action against DPP4 enzyme.

These mechanisms may have important clinical implications for individuals with impaired glycaemic control, by preventing or slowing carbohydrate digestion [4-8] and

preserving biologically active incretins to stimulate glucose-dependent insulin secretion in the body thereby improving glycaemic control post-meal [9, 10, 30, 31].

Grape seed was shown to be a good inhibitor of α-amylase in the present study. The α-amylase inhibitory activity of grape seed has been attributed to the procyanidins (condensed tannins) abundantly present in grape seed, with increasing polymerisation of the procyanidin producing a higher inhibition on α-amylase [35, 37]. This finding was in agreement with previous studies showing grape seed to be an inhibitor of α-amylase [33, 34, 36]. The inhibitory action of grape seed was further ascertained by an acute human study demonstrating postprandial glucose reduction in healthy participants after a high carbohydrate meal, elucidating the inhibition of α-amylase as one of the underlying mechanisms for attenuating glucose response [55].

Rooibos tea extract showed good inhibition of α-amylase in the present study, which was in agreement with an *in vitro* study demonstrating inhibition on porcine α-amylase (IC_{50} of 18 mg/mL), with subsequent attenuation in postprandial glucose in a mice model [38].

For this study olive leaf extract showed minimal inhibition on α-amylase. Olive leaf has been shown to be a weak inhibitor of α-amylase [40]. This is with agreement with Kerimi and colleagues (2018) who demonstrated weak inhibitory effect of olive leaf on human salivary α-amylase (IC_{50} of 0.8 mg/mL), with no significant postprandial glucose responses in healthy participants [43]. However, other studies have ascertained the inhibition of olive leaf on several digestive enzymes such as α-amylase [41, 46, 47]. One study attributed glycaemic improvement in borderline diabetic participants to the inhibitory effects of olive leaf on human pancreatic α-amylase, with luteolin and oleanolic acid identified as responsible for α-amylase inhibition [42]. Nonetheless, there were other studies conducted by Pyner, Kerimi and co-workers elucidating that olive leaf might be more effective in inhibiting sucrase instead, potentially leading to reduced sucrose digestion in humans [43-45]. There is therefore evidence that olive leaf exhibits inhibition on α-amylase. However, differences in extraction methods, extract sources and concentrations, as well as enzyme origins [7, 36, 51, 56, 57] used might have resulted in outcome variations observed between the present study and previous studies.

The New Zealand pine bark extract exhibited considerable inhibition against α-amylase (IC_{50} of 3.98 mg/mL) in the present study. Similarly, a Korean pine bark extract also showed inhibitory potency against α-amylase (IC_{50} of 1.69 μg/mL), although the exact phenolic composition that attributed to its inhibitory action was not known [49].

In the present study grape seed, rooibos tea and New Zealand pine bark extracts have demonstrated relatively strong inhibition of DPP4 enzyme, and their DPP4 inhibition was greater compared to their inhibition on α-amylase at the same concentration of 5 mg/mL. The inhibitory action of the pine bark extract on DPP4 (IC_{50} of 2.51 mg/mL) suggesting an indirect increase in active GLP-1 levels may help explain the improved postprandial glucose responses ($p<0.05$) seen in healthy participants in our previous study [32]. Likewise, the inhibition on DPP4 enzyme by grape seed and rooibos tea extracts may also support the improvements observed in postprandial glycaemic responses and insulin sensitivity ($p<0.05$) in participants with prediabetes conducted by our group (unpublished). The present findings were also supported by *in vivo* studies that demonstrated that grape seed extract was able to inhibit intestinal DPP4 enzyme resulting in an increase in active GLP-1 levels in rats [52, 53]. In our previous study, olive leaf extract was shown to increase mean incremental area under the curve of insulin ($iAUC_{insulin}$) in participants with prediabetes (16.7% increase, $p=0.040$) (unpublished). However as olive leaf has demonstrated negligible inhibitory action on enzymes in the present study, other underlying mechanistic actions that caused an increase in insulin secretion in the participants should be further explored.

The merits of the present study included understanding the inhibitory action of grape seed, rooibos tea, olive leaf and pine bark extracts on enzymes such as α-amylase and DPP4 involved in regulating postprandial glycaemic control. Moreover, this study has also helped to elucidate the inhibition of DPP4 enzyme as one of the potential underlying mechanistic actions in enhancing the incretin effect to improve glycaemia in the two human studies (Pine Bark study and GLARE study) conducted by our group.

However, there are some limitations to the study. The IC_{50} values of the plant extracts except for pine bark have not been measured, making comparisons difficult with other similar studies that have reported IC_{50} values.

This study was only a preliminary *in vitro* study to determine the presence of inhibitory capabilities of grape seed, rooibos tea, olive leaf and pine bark extracts. The *in vitro* outcomes were based on the un-metabolised forms of the plant extracts interacting with, and inhibiting the α-amylase and DPP4 enzymes, rather than their active metabolites typically produced in the body after being metabolised. This may therefore not translate into equivalent outcomes in humans, given the knowledge that plant polyphenols undergo extensive metabolism in the body that could alter their enzymatic inhibitory effects [58-62]. For example, metabolites of grape seed were not shown to inhibit plasma DPP4 enzyme, suggesting that the metabolised forms of grape seed reaching the systemic circulation were

not effective in inhibiting DPP4 [52]. It may therefore be more useful to identify and purify active metabolites of the extracts of interest and subject them to similar α-amylase and DPP4 inhibitory assays in order to obtain outcomes that are more representative of human physiology [61, 63].

In addition, doses used in the present *in vitro* study were greater than would be for a normal intake, and hence may not reflect the actual plasma concentrations of the extract metabolites, which rarely exceed nanomoles per litre (nmol/L), that are available to potentially inhibit α-amylase and DPP4 enzymes in the body [61, 62].

Even though the present study has used recombinant human DPP4 enzyme that is highly relevant to human physiology for the *in vitro* DPP4 inhibitory assay, this occurred in a cell-free environment. It was shown that phenolic compounds such as flavonoids or grape seed was able to more strongly inhibit recombinant human DPP4 in a cell-free assay but exhibited weak to negligible inhibitory activity on DPP4 isolated from human plasma, intestinal CaCo2 cells, or saliva [52, 64]. One of the reasons might be that polyphenols such as flavonoids were hindered from inhibiting the DPP4 enzyme due to interactions with proteins naturally present within the blood such as human serum albumin being most abundant, rendering them unavailable to bind to DPP4 [64]. It was suggested that DPP4 enzymes obtained from whole blood or plasma should be used as the enzyme source for the assay. This would provide better physiological resemblance and a more accurate assay result when determining potential inhibitory effects of polyphenols *in vivo* [65]. However, it may be challenging to obtain sufficient DPP4 in the plasma due to low circulating concentrations [66].

Sources of human DPP4 enzymes may also play a role in influencing the inhibitory activity of plant extracts on DPP4 enzyme. DPP4 enzyme exists as two forms within the body; one being the soluble form in the blood, and the other membrane-bound on the luminal surface of the vascular endothelium [65]. Although these two forms may have identical catalytic regions to interact with polyphenols, they are not 100% structurally similar [66]. This may therefore result in varying inhibitory efficacies depending on which form and enzymatic site of the DPP4 the polyphenols interact with within the body [65]. For example, 1 g grape seed/ kg body weight treatment was shown to reduce intestinal DPP4 enzyme by 34.3% but showed negligible inhibitory activity with blood plasma soluble DPP4 in a rat model [52]. It was likely either due to the generally low bioavailability of polyphenols in the body to be absorbed into the bloodstream, or that grape seed metabolites in the plasma would have been combined with proteins such as albumin rendering them less available to interact

and inhibit DPP4 [64]. Studies have elucidated that the inhibition of intestinal DPP4 rather than its circulating, soluble form would be more relevant to incretin and glucose metabolism [52, 67-70]. This is because DPP4 activity proximate to incretin production site may have a greater impact on glycaemic control when inhibited, as rapid degradation of incretins already occurs prior to entering systemic circulation, and that soluble plasma DPP4 only constitutes a small proportion of the DPP4 concentration in the body [52, 67-70]. Therefore, it is noteworthy for future studies that the inhibitory efficacy of plant extracts may differ depending on the target site of DPP4 inhibition, whether using human caco-2 cells for intestinal inhibition, or *ex vivo* human plasma for plasma inhibition, although intestinal inhibition of DPP4 may be more relevant for improving glucose homeostasis.

It has also been demonstrated that different substrates used for the DPP4 assay may generate differing degrees of inhibitory outcomes. The present study has used H-Gly-Pro-AMC, which was a fluorometric substrate due to its higher sensitivity for biological samples [64]. It was shown that using the fluorometric substrate may generate 10-fold higher inhibitory activity on DPP4 enzyme than a colorimetric substrate (Gly-Pro-p-nitroanilide hydrochloride), but a luminescent substrate (Gly-Pro-aminoluciferin) would produce an even higher inhibitory activity compared to the fluorometric substrate [71]. Hence, there is a possibility that polyphenols undergoing the DPP4 enzyme inhibition assay may also interact with the colorigenic, fluorogenic and luminescent substrates rather than the DPP4 enzyme to alter assay sensitivity [65]. To detect any unwanted interaction between polyphenols in the DDP4 inhibition assay it is important to include a control without presence of polyphenols [65]. It is also vital to take note of the specific substrate used for the DPP4 inhibition assay when comparing between plant extracts and other similar studies.

Similarly, the α-amylase assay using DNS reagent has been widely used to investigate the inhibition of compounds including polyphenols on the α-amylase enzyme. However, polyphenols such as epigallocatechin gallate (EGCG), gallic acid, phlorizin, have been demonstrated to interfere with the assay by interacting with the DNS reagent [7]. It was suggested that the interference increased with the number of hydroxyl (OH) groups present in the phenolic structures [7, 72]. Therefore the removal of various polyphenols that have been shown to interfere with the assay, for example by using solid phase extraction [73], should be considered in pre-tests if DNS reagent is to be used. However, this may prove to be a challenge as plant extracts predominantly contain polyphenols that contribute to the inhibitory effects observed on α-amylase [4-8]. An optimised α-amylase assay is thus warranted.

Additionally, porcine-derived α-amylase was used in the present study, where the amino acid composition of the porcine α-amylase is 14% different to the human α-amylase leading to functional differences [7, 74]. Therefore, using human salivary α-amylase may be a better representation of human physiological inhibition of α-amylase [73]. As differences in enzyme sources can play a part in different inhibitory outcomes, this should be taken into account when extrapolating the *in vitro* results to explain the possible underlying mechanisms of plant extracts in impacting glycaemia in humans [7, 56].

Considering that the enzyme inhibition is only effective during a meal, their bioavailability and subsequent functionality may also be influenced by several dietary factors such as presence of macronutrients [63, 75, 76]. Therefore, the present findings on the mechanistic actions of enzyme inhibition should be further validated in humans. The inhibition of the plant extracts on α-amylase could be confirmed in hydrogen tests [77] and ^{13}C breath tests [78-80] in humans to quantify degree of carbohydrate malabsorption. Similarly, the inhibition of DPP4 enzyme can be ascertained by measuring concentrations of active circulating incretins (e.g. GLP-1) in plasma samples collected from participants.

6.5 Conclusions

Extracts of grape seed, rooibos tea, and New Zealand pine bark are capable of inhibiting α-amylase and DPP4 enzymes. Their role in inhibiting these enzymes may be one of the mechanisms in which these extracts improved glycaemic responses in humans. Further human studies are warranted to validate the underlying hypoglycaemic mechanisms of action of these plant extracts.

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Chapter 7
Discussion and conclusions

This chapter provides an overview of findings from this PhD study with the assessment of strengths and limitations, followed by outline for future research and overall conclusion.

7.1 Summary of key findings

This chapter discusses the main findings of the investigation of the hypoglycaemic impact of the antioxidant-rich plant extracts (New Zealand pine bark, grape seed, rooibos tea and olive leaf) in an acute trial in both healthy participants (Pine Bark study) and participants with prediabetes (GLARE study) living in New Zealand. A summary of the key findings aligned with the four Research Questions formulated for this PhD Study (Chapter 1) are outlined below:

1. **Do plant extracts improve postprandial glycaemia in individuals with prediabetes?**
   Human clinical trials examined in the narrative review (Chapter 3) demonstrated that a number of plant extracts have hypoglycaemic potential and improved glycaemic responses in people with prediabetes. More interestingly when participants were grouped by prediabetes subgroup types (IFG, IGT and IFG/IGT), some plant extracts exhibited preferential improvements in either fasting or postprandial glycaemic indices. This means not all plant extracts exert the same impact on glycaemic control and that some extracts may be more effective in improving IFG or IGT, respectively, with IFG/IGT benefiting from either improvement.
2. **Does New Zealand pine bark improve postprandial glycaemia in healthy individuals?**
   Primary analysis showed no significant change in glucose incremental area under the curve (iAUC) with the lower dose (50 mg) (p=0.123, 13.4% reduction) but a significant reduction in $iAUC_{glucose}$ with the higher dose (400 mg) (p=0.016, 21.3% reduction) compared to control in healthy participants. Secondary analysis with stratification showed that New Zealand pine bark improved $iAUC_{glucose}$ in both doses (50 and 400 mg) compared to control only in participants exhibiting monophasic glucose curve shapes. The higher dose (400 mg) also improved other glycaemic indices such as percentage increment of postprandial glucose (% PG), glucose peak, and 2h postprandial glucose (2hPG) in monophasic participants.
3. **Do extracts of grape seed, rooibos tea and olive leaf improve postprandial glycaemia in individuals with prediabetes?**
   Primary overall analysis showed no significant differences in glucose and insulin indices between plant extracts and control in participants with prediabetes (p>0.05).

Secondary analysis with stratification demonstrated that all plant extracts showed improvements in various glycaemic measures compared to control in participants with prediabetes who had a less healthy metabolic profile of delayed glucose and insulin peak times.

4. **What are the underlying hypoglycaemic mechanisms of these plant extracts on improving glucose homeostasis?**
   The outcome of the mechanistic study elucidated the presence of potential inhibitory action of the New Zealand pine bark, grape seed and rooibos tea on digestive enzyme (α-amylase) and dipeptidyl-peptidase-4 (DPP4) enzyme.

7.2 Discussion of main findings

The investigation of the hypoglycaemic potential of four antioxidant-rich plant extracts (New Zealand pine bark, grape seed, rooibos tea and olive leaf) in the Pine Bark study and the GLARE study elucidated the hypoglycaemic effects of the extracts with improvements in a range of glycaemic measures.

The narrative review (Chapter 3) has set the stage in understanding the impact of plant extracts on glycaemia in individuals with prediabetes. Eleven human clinical trials including two acute studies and the rest being chronic studies and involving a total of eight different plant extracts demonstrated promising hypoglycaemic potential of the plant extracts in the prediabetes cohort. Further examination of the impact of plant extracts on the subgroups of prediabetes showed plant extracts such as *Artemisia princes* Pampanini [1, 2], soy (Glycine max (L.) Merrill) leaf [3, 4] and *Citrus junos* Tanaka peel [5] have been shown to improve fasting glycaemia and thus may benefit individuals with IFG. In contrast, white mulberry (*Morus alba* Linn.) leaf [6-8], persimmon (*Diospyros kaki*) leaf [9] and *Acacia. Mearnsii* bark [10] were shown to improve postprandial glycaemia and hence may be more beneficial for individuals with IGT. *Elaeis guineensis* leaf was observed to improve both fasting and postprandial glycaemic measures depending on the concentration used [11], and hence may be useful for all prediabetes subgroups. It follows that treatments should be made available that are specific for each of the subgroup to obtain optimal glycaemic outcomes. However, more studies are required to ascertain the usefulness and practicality of giving targeted interventions to individuals with respect to their prediabetes subgroups.

Therefore, an important aspect of the PhD work was to understand the impact of intervention with plant extracts on the existent subcategories of impaired glycaemic control

in humans. A decision was made to include participant stratification in the data analysis of both the Pine Bark study and the GLARE study following growing research corroborating the existence of heterogeneity in impaired glycaemic control due to varying metabolic profiles that resulted in high variability in inter-individual responses to interventions [12-17]. This phenomenon was demonstrated in both the Pine Bark study and the GLARE study that individuals with worsening glycaemic control or metabolic profiles responded significantly better to intervention. Faerch and colleagues (2013) as well as Krishnan and co-workers (2012) have also advocated the importance of stratification in studies based on different metabolic profiles to determine the efficacy of interventions [13, 18]. This method of analysis by stratification based on glycaemic profiles was in line with other nutrition intervention studies that have explored secondary analysis of participant stratification, based on for example, delayed glucose peaks [19], differential prediabetes subgroups [20], and degrees of fasting glucose [21, 22], and observed differences particularly in participants with worsening clinical outcomes.

The differential degrees of glycaemic control in participants have shown varying responses to intervention in both the Pine Bark study (chapter 4) [23] and the GLARE study (chapter 5). Therefore stratification based on the different metabolic profiles was important in order to elucidate the impact of intervention with plant extracts on both responders and non-responders [12, 13, 19, 23, 24].

The Pine Bark study suggests that the New Zealand pine bark may have hypoglycaemic effects in healthy participants, particularly those exhibiting monophasic glucose curve shapes. The stratification of participants based on postprandial glucose curve shapes was first introduced by Tschritter and group [25], where monophasic shapes were associated with poorer glycaemic control and an increased risk of T2DM compared to complex shapes (biphasic or triphasic) [25-29]. Significant improvements in $iAUC_{glucose}$ were observed in both doses 50 mg (p=0.034) and 400 mg (p=0.012) in the monophasic subgroup compared to control. The higher dose (400 mg) also significantly improved other glycaemic indices such as %PG (p=0.010), glucose peak (p=0.025) and 2hPG (p=0.027) indicating improved glycaemic control in this group [30-32]. The study elucidated the importance of examining effectiveness of intervention in participants with different postprandial glucose curve shapes, even though being normoglycaemic and supposedly healthy. This also highlights that pine bark should be further examined in a longer-term human study, after having observed its acute impact on glycaemic control in healthy participants. The outcomes of the Pine Bark study were also in agreement with other studies on the French Maritime pine

bark that have been extensively studied. Studies on the French pine bark (100 to 300 mg/day) showed improvements in glycaemic responses in humans [33-38]. However, all the studies on French pine bark were chronic studies (three weeks to six months) in individuals who were healthy, having T2DM or metabolic syndrome, and were different to the participant demographics and study duration in the Pine Bark study. Further studies are warranted to examine the effect on other measures, such as insulin on a longer-term basis, and in other population groups, such as those with prediabetes or T2DM who may be in greater need of intervention.

The GLARE study has elucidated the hypoglycaemic effects of grape seed, rooibos tea and olive leaf extracts in participants with prediabetes, particularly those exhibiting delayed glucose and insulin responses (less healthy subgroup), which indicated poorer glycaemic control. The stratification was based on the methodology developed by Takahashi and colleagues (2018) [39]. A delay in insulin peak or a loss of early phase insulin response is often a glucose metabolic defect associated with impaired glycaemic control [40, 41], as an early phase insulin response is important to suppress glucagon release and inhibit hepatic glucose production [42-45]. The stratification based on glucose and insulin peak times following the Takahashi et al. (2018) method was employed in the GLARE study instead of the stratification using postprandial glucose curve shapes used in the Pine Bark study for two main reasons. Firstly, participants with prediabetes have a higher variability in response to OGTT [46-50], compared to healthy participants [51], that could result in the glucose shapes being less reproducible [28, 52, 53]. The decreased reproducibility might introduce the risk of misclassifying participants into the wrong glucose curve shape group. Secondly, postprandial glucose curve shapes do not take into account insulin responses, which are important parameters of glycaemia. Hence, it was more favourable to employ the stratification based on the Takahashi et al. (2018) method in the GLARE study.

In the less healthy subgroup, grape seed consumption was shown to improve various glucose and insulin indices such as significant reductions in 2hPG (p=0.034) and 2hPI (p=0.029), with significant improvements in mean $ISI_{overall}$ (p=0.028) and MCR (p=0.016) compared to control. Grape seed also showed a trend towards improvement in time to insulin peak in the less healthy subgroup (p=0.054) and early phase insulin response measured by mean ISI_{first} (p=0.082) and IGI_{30} (p=0.078), an indication of acute restoration of early phase insulin secretion and a possible amplification of early insulin response via incretin effect to aid glycaemic control [54]. This finding agrees with previous acute RCTs demonstrating grape seed extracts of various concentrations (100-500 mg) to improve markers of glucose

metabolism such as $iAUC_{glucose}$ in healthy individuals or those with metabolic syndrome [55-57]. However, the findings were in contrast to other chronic trials on healthy participants, those with T2DM or metabolic syndrome that showed no significant improvement in fasting blood glucose (FBG), glycated haemoglobin A1c (HbA1c) or fasting insulin (FI) with grape seed extract (150-400 mg/day) [58-61]. Differences in the extract grade, doses, small sample sizes and study duration might explain the inconsistencies in results. Furthermore, most of the previous studies on grape seed extract have not measured changes in insulin outcomes, making comparisons with GLARE study difficult.

Rooibos tea extract was shown to improve acute β-cell function as observed in an improved oral disposition index (DI) (p=0.031) and a trend towards improvement in insulin-secretion-sensitivity index-2 (ISSI-2) (p=0.074) in the less healthy subgroup. These outcomes were indicative of improved glycaemic control and suggest the potential to restore β-cell function [62-65]. A previous study conducted by Chepulis et al. (2016) showed significant reduction in $iAUC_{glucose}$ in healthy participants after consuming 760 mg (≥30% polyphenols) of rooibos tea extract [56], in contrast to the GLARE study where rooibos tea extract (1,714 mg, 485 mg total polyphenols) had no significant improvement in $iAUC_{glucose}$ and 2hPG in participants with prediabetes. The difference in results might be due to the metabolic differences in the participants in the two studies, with a much higher dose required for participants with prediabetes to elicit a significant reduction in $iAUC_{glucose}$.

Although olive leaf did not demonstrate any improvement in postprandial glucose responses, it exhibited a trend toward improvement in various insulin sensitivity measures such as mean IGI_{30} (p=0.078), ISI_{first} (p=0.075) and ISI_{second} (p=0.062) in the less healthy subgroup compared to control. The improvement in early phase insulin responses might be associated with amplification of the incretin effect in this subgroup of participants [54]. The improvement in oral disposition index (IGI_{30}) was in agreement with the study conducted by de Bock and colleagues (2013) that also demonstrated significant improvement in IGI_{30} in non-diabetic, overweight men, which was an indication of enhanced β-cell function, after a chronic 12-week consumption of olive leaf extract (51.1 mg oleuropein, 9.7 mg hydroxytyrosol/day) [66]. Previous chronic studies of similar design have shown beneficial effects in glycaemia such as HbA1c, FI, and area under the curve (AUC) of glucose and insulin in healthy or T2DM participants after olive leaf consumption [66, 67]. An acute study conducted by Komaki and colleagues (2003) also demonstrated significant postprandial glucose (PG) reduction in borderline diabetic subgroup of participants [24]. A 12-week study on olive leaf tea consumption (32.4 mg/100 g oleuropein, 1.2 mg/100 g hydroxytyrosol in

330 mL of tea beverage three times a day) also showed significant reduction in FBG in participants with prediabetes, although no significant changes were seen in HbA1c, FI, and homeostatic model assessment of insulin resistance (HOMA-IR) [68].

On the contrary, other chronic and acute studies did not show significant improvements in glycaemic responses such as FBG, insulin, HOMA-IR, and PG in healthy participants or those with metabolic syndrome [69-71]. Again, the extract types and varying doses, study duration and metabolic profile of participants might have contributed to the differences in results observed.

Olive leaf extract was also shown to significantly elevate insulin levels in the study without significantly altering postprandial glucose levels, which could partly explain the improvement in IGI_{30}. The increase in insulin levels with olive leaf might be attributed to its ability as an insulin secretagogue in *in vitro* and animal studies [72, 73]. Further human studies are required to determine the effects of olive leaf extract on insulin levels in the prediabetes cohort.

The post-stratification analysis showed no significant improvements in glycaemic measures in the healthier subgroup. The outcomes of the GLARE study therefore emphasised the importance of investigating the impact of intervention based on the different metabolic profiles of individuals, which concurs with the outcome of the narrative review in Chapter 3.

Multiple underlying mechanisms of action in glucose metabolism might have played a crucial role in the observed improvements in glycaemic response in both the Pine Bark study and the GLARE study. Therefore an *in vitro* mechanistic study was undertaken to explore the potential inhibition of the New Zealand pine bark, grape seed, rooibos tea and olive leaf extracts on two key enzymes (α-amylase and DPP4 enzymes) as one of the possible mechanistic actions leading to improved glycaemic outcomes.

The mechanistic study (Chapter 6) demonstrated that the New Zealand pine bark, grape seed and rooibos tea extracts possessed inhibitory action on digestive enzyme α-amylase and DPP4 enzyme to various degrees. The New Zealand pine bark exhibited the greatest inhibitory effects against both enzymes compared to the other extracts. The ability to inhibit α-amylase with the ingestion of the extracts at mealtimes suggests a possibility of delayed carbohydrate digestion into glucose for absorption [74-78], and potential enhanced incretin effect via DPP4 enzyme inhibition [79-82]. However, although the results of the inhibition of α-amylase could not be used to explain the observed glycaemic improvements in both the Pine Bark study and the GLARE study as liquid glucose was used in the oral glucose tolerance test (OGTT), the outcomes can serve to inform future studies using mixed meal as a

carbohydrate load. The inhibitory action of the pine bark extract on DPP4 enzyme suggests that an indirect increase in active GLP-1 levels may help explain the improved postprandial glucose responses ($p<0.05$) seen in healthy participants in our Pine Bark study [23]. Likewise, the inhibition on DPP4 enzyme by grape seed and rooibos tea extracts may also support the significant improvements observed in postprandial glycaemic responses and insulin sensitivity in participants with prediabetes in the GLARE study.

In contrast, olive leaf extract showed minimal inhibition on α-amylase and no inhibition action against DPP4 enzyme. Although olive leaf extract was found to inhibit digestive enzymes such as α-amylase in other studies [24, 67, 71, 83-87], the current mechanistic study showed negligible inhibition on the enzymes tested, suggesting that differences in extraction methods, extract sources and concentrations, as well as enzyme origins [77, 88-91] used might have resulted in outcome variations observed. In the GLARE study olive leaf extract was shown to increase mean $iAUC_{insulin}$ in participants with prediabetes (16.7% increase, $p=0.040$). Other underlying mechanisms of action might be responsible for the observed increased in insulin levels, such as olive leaf being an insulin secretagogue and promoting insulin secretion [72, 73].

Similar to other intervention study procedures, the Pine Bark study and the GLARE study were with short-term, acute trials to determine the initial primary intervention outcomes before proceeding into chronic trials. Therefore, the study outcomes from both trials might differ in comparison to chronic studies looking at sustainability of effect of the plant extracts under daily life conditions. Conducting chronic trials on plant extracts is especially important as there is yet insufficient data regarding their storage in the body to have a prolonged impact on glucose metabolism. Research has shown that polyphenol metabolites of plant extracts in the body reach maximal plasma concentrations within 1-3 h [92-94], with some taking 5-7 h, or up to 24 h [95-98]. The elimination half-life of polyphenols is 1-18 h, but with most polyphenols excreted in less than 8h [99]. The quick passage through the body may undermine the potential impact of plant extracts on glycaemia. This may explain why the abovementioned chronic studies that investigated the New Zealand pine bark, grape seed, rooibos tea and olive leaf that showed inconsistent results concerning their sustained effects on glycaemic responses. Nonetheless, the slower rate of elimination for some polyphenols may indicate the possibility that high plasma concentrations could be maintained with regular consumption of phenolic-rich plant extracts [100, 101], that can elicit small but appreciable impact on glycaemic control. Therefore the New Zealand pine bark, grape seed, rooibos tea

and olive leaf warrant further investigation in chronic studies in the prediabetes cohort to determine their prolonged hypoglycaemic effects.

7.3 Strengths

The strengths of the PhD study included the use of a robust crossover design where participants were their own control and underwent each of the treatments, and therefore a smaller sample size was required to reach a similar level of significance in both the Pine Bark study and the GLARE study [102, 103]. Based on previous work a prospective power calculation was done so that sufficient sample size was determined to see a potential difference in each study.

The measurement of HbA1c has been the recommended screening tool for prediabetes because it a quick test that measures blood glucose levels without the need to undergo fasting, unlike FBG or 2hPG measurement [104]. HbA1c gives an indication of chronic glycaemic control by measuring average blood glucose value over several months (approximately 2-3 months) without confounding factors such as acute perturbations from stress, diet and exercise [105], and therefore may confer a higher reproducibility compared to FBG or 2hPG [106]. Therefore, HbA1c was used as a screening tool in the GLARE study recruitment. In the Pine Bark study, both HbA1c and FBG were taken for healthy participants as recommended in research to use more than one test to ascertain glycaemic status [107, 108].

The plant extracts investigated in both the Pine Bark study and the GLARE study were commercially manufactured based on Good Manufacturing Practice (GMP) and have been characterised and standardised with respect to their main bioactive components for research purposes. The plant extracts in the GLARE study were also standardised based on their total antioxidant capacity (TAC) using a well-evaluated method of Oxygen Radical Absorbance Capacity (ORAC) [109, 110], and therefore efficacy could be compared amongst the extracts.

An important merit from both clinical trials was the use of multiple glycaemic indices to determine various potential specific changes in glucose (glucose peak, glucose peak time, %PG, MCR), insulin measures such as overall insulin sensitivity (Matsuda index (ISI/M), oral glucose insulin sensitivity (OGIS), Stumvoll $ISI_{overall}$), first and second phase insulin sensitivity indices (ISI_{first}, ISI_{second} and IGI_{30}), and β-cell function (ISSI-2, DI) in response to the plant extracts.

7.4 Limitations

The main limitations of the Pine Bark study included not having measurements of insulin or C-peptide and insulin sensitivity to ascertain their changes and effect on glycaemia. A smaller sample size after stratification of participants into two distinct glucose curve shapes was another limitation of the Pine Bark study. Although a prospective power calculation was done, this did not take into account the stratification of participants into subgroups.

One of the challenges encountered in the GLARE study was the prolonged recruitment of participants with prediabetes. The initial plan was to contact general practitioners (GPs) for collaboration in order to obtain eligible participants with prediabetes via enrolment in primary care. Although several GPs were keen to collaborate to help recruit their patients who had prediabetes, it was unfortunate that approval to proceed was not given by the Procare Health Limited that was part of the New Zealand Primary Health Organisation (NZ PHO). Other GPs that were contacted had a lack of funding and manpower for the additional collaborative work. There was also a general lack of awareness regarding the existence of prediabetes within the population, which has prevented eligible individuals from being interested and enrolling in the study. This has led to the slower than expected recruitment of eligible participants for the GLARE study. The GLARE data collection took approximately two years for recruitment, where n=130 participants were assessed for eligibility but n=104 of them were excluded due to having lower than required HbA1c, being diabetic or taking metformin or glucose control medications.

Moreover, due to the higher prevalence of prediabetes in the older population [111-113], overweight and obese cohort [112, 114], the GLARE study recruited older participants (mean age 65.0 ± 1.6 years) as well as overweight participants (body mass index (BMI) 27.3 ± 1.1 kg/m^2). However, this population group has led some participants to encounter difficulty in cannulation of the antecubital fossa region of the arm, with 34.6% of the participants withdrawn from the study. There were n=7 participants who withdrew after the first trial visit, and n=2 participants who dropped out after the third trial visit.

Nonetheless, another initiative to collaborate with Auckland Diabetes Association Mobile Diabetes Awareness service to recruit eligible participants from the public, along with the continued interest of past participants from previous clinical trials, meant sufficient participants were recruited and the study was successfully completed and data analysed.

A further challenge was the disruption to a part of the GLARE study experiment involving the analysis of incretin levels in the samples collected to quantify the incretin effect due to the COVID-19 pandemic. Hence, the incretin concentrations could not be determined in this PhD study.

The study limitations of the GLARE study included not having a larger sample size to account for participant stratification into different glucose and insulin peak times using the Takahashi et al. (2018) method [39]. In hindsight it would have also been useful to have a large enough sample size to enable the sub-classification of participants into prediabetes subgroups (IFG, IGT and IFG/IGT) to elucidate the impact of intervention on each subgroup. A larger sample size could also help account for gender differences in response to intervention, as studies have shown that men and women exhibit different pathogenesis of T2DM [115-117].

Although HbA1c measurement was the NZSSD recommended method to screen for prediabetes in the GLARE study [118], it has certain limitations. HbA1c values can be influenced by ethnicity, age, gender, FBG, BMI, and haemoglobin related conditions, and other biological determinants of haemoglobin glycation [108, 119-121]. HbA1c may also misclassify or underestimate prediabetes prevalence [104, 122, 123], due to the reduced sensitivity of HbA1c and/or specificity to detect prediabetes that is characterised by low to intermediate levels of dysglycaemia [123, 124]. Therefore, it is recommended that HbA1c value be taken in conjunction with FBG or 2hPG [107, 108, 123]. However, a confirmatory test with either FBG or 2hPG for prediabetes in addition to HbA1c has not been carried out in the GLARE study. This has led to n=13 participants in the GLARE study having an elevated HbA1c value but exhibiting normal FBG and 2hPG based on baseline measurements at the control visit. It was evident that there was discordance with other glycaemic measures such as FBG and 2hPG that are equally important clinical outcome indicators of glycaemic status [125], and that HbA1c measured different aspects of glycaemia compared to FBG or 2hPG [108, 124, 126]. Therefore it is crucial that a combination of glycaemic measures (HbA1c, FBG and 2hPG) be taken in order to have a more accurate quantification of prediabetes status of the participants.

Furthermore, HbA1c measurement was also unable to distinguish between pathophysiologically different prediabetes subgroups: IFG and IGT [127], resulting in a heterogeneous mixture of participants with various glycaemic profiles in the GLARE study with participants having IFG (n=2), IGT (n=1) and IFG/IGT (n=3), in addition to having an elevated HbA1c value. This phenomenon was also evident in several other studies [128-130].

Some study participants (n=6), who at the point of screening, had HbA1c values (38-40 mmol/mol) below the prediabetic range of 41-49 mmol/mol [118]. However, the participants were recruited based on having at least one risk factor for T2DM (overweight or obese, high blood pressure, prediabetes history, and family history of T2DM or cardiovascular disease (CVD)). A study conducted by Marini et al. (2014) elucidated that the offspring of diabetic parents (one of T2DM risk factors) and having HbA1c values above 39 mmol/mol (5.7%) were already suffering from significant faltering insulin sensitivity and β-cell function [131]. Edelman et al. (2004) in their research also recommended closer scrutiny for patients with prediabetes having baseline high-normal HbA1c (37.7-42.1 mmol/mol, 5.6-6.0%) to elevated HbA1c (43.2-51.9 mmol/mol, 6.1-6.9%) especially if they were obese, as their diabetes incidence were higher each year, 2.5% and 7.8%, respectively [132]. Therefore it was found justifiable to include participants with lower HbA1c values in the GLARE study.

The GLARE participants who took part in the study only had borderline prediabetes (mean HbA1c 42 ± 1 mmol/mol), and therefore the study outcomes might differ from those with more severe prediabetic status with a higher HbA1c value. Other factors such as ethnicity [133, 134], age [111-113], gender [115-117], abdominal and visceral obesity [114, 135] may also produce different study outcomes and warrant further study.

The plasma insulin was collected and analysed in the GLARE study to investigate insulin responses to the plant extracts. However, insulin concentrations in contrast to C-peptide, might be influenced by its pulsatile nature of secretion, where it undergoes first-pass metabolism in the liver where insulin is cleared from the bloodstream [136]. In addition, individuals have been shown to exhibit different rates of insulin clearance resulting in different levels of circulating insulin in the blood [137]. These factors might increase the variability in insulin concentrations collected in the study.

The reproducibility of the stratification using postprandial glucose curve shapes for the Pine Bark study and glucose and insulin peak times in the GLARE study has yet to be examined. Misclassification may take place where participants do not fit into a certain preset definition of the stratification. However, the postprandial glucose curve shapes (Pine Bark study) and the glucose and insulin peak times of participants (GLARE study) were plotted and checked against to confirm that there was no misclassification of participants. Furthermore, as the sample size was small in both studies, the probability of misclassifying was greatly reduced.

Both the Pine Bark study and the GLARE study employed the OGTT with liquid glucose as the carbohydrate load for the measurement of postprandial glycaemic excursions.

The primary reason for choice was because OGTT was a simple, gold standard model to understand the impact of plant extracts on glycaemia without the interferences from other nutrients such as fats and proteins found in a mixed meal [138, 139]. A study conducted by Meier and colleagues (2009) has also demonstrated that an OGTT was significantly correlated to a standardised mixed meal [140]. Therefore, the OGTT suited the purposes of the Pine Bark study and the GLARE study. Nonetheless, a mixed meal is typically used to represent normal food intake and studies have shown that in prediabetes and T2DM glycaemic measures such as insulin, glucagon, β-cell function and incretin might have altered responses depending on the type of meal consumed along with the intervention [140-144]. Glucose and insulin responses may also differ depending on the polyphenol-carbohydrate combination present, particularly when a plant extract is consumed together with a carbohydrate load [145]. In addition, research has also elucidated the higher variability and decreased reproducibility of the OGTT procedure, with individuals transitioning from normoglycaemia into having impaired glycaemic control tending to have a higher intra-individual variability in response to an OGTT [46-50]. Mixed meal tolerance test (MMTT) has been shown to have good repeatability for glucose and insulin responses from healthy individuals to those with prediabetes and diabetes [146, 147]. Hence, future studies may explore the use of mixed meal in the longer-term study of plant extracts on glycaemic responses in humans.

The bioavailability of the extracts in the body has not been investigated to understand the degree of metabolism and post-metabolism hypoglycaemic efficacy of the extracts, especially in participants with poor glycaemic control. Therefore it is an area of potential research in the future to quantify the postprandial metabolites of plant extracts to determine the effective doses required to improve glycaemic responses in humans.

The limitations of the mechanistic study included only examining only a small aspect of various mechanistic actions (enzyme inhibition on α-amylase and DPP4) displayed by plant extracts to impact glycaemic control and other mechanisms should also be explored.

7.5 Future directions

The current PhD work has identified a few recommendations for further research in the area of the hypoglycaemic impact of plant extracts on impaired glycaemic control, or prediabetes:

1. Acute and chronic human studies of the impact of the New Zealand pine bark on glycaemic responses in those with prediabetes is required, with the inclusion of measurements such as insulin, C-peptide, insulin sensitivity, and β-cell function (e.g., HOMA-IR, Matsuda index, and other glycaemic indices).
2. A chronic human study (of at least 12 weeks, which is the minimum duration required to reliably detect sustained effect of dietary intervention [148]) of the impact of grape seed, rooibos tea and olive leaf extracts of similar or higher dose on individuals with prediabetes is warranted. Moreover, recruiting a larger sample size to account for stratification of participants with prediabetes into their subgroups (IFG, IGT and IFG/IGT) is recommended.
3. Future research extending from the GLARE study could also look into the impact of intervention with plant extracts on hyperinsulinaemia as an independent risk for T2DM in participants with prediabetes [149-152]. Individuals with impaired glucose control may also suffer from an overall higher insulin secretion than both normal glucose tolerant and diabetic individuals [44, 153, 154]. Future studies could employ the methodology established by Hayashi et al. (2013) [155] and Crofts and colleagues (2016) [156] to determine hyperinsulinaemia in participants with prediabetes [157].
4. Future human studies on prediabetes should employ more than a single test (HbA1c, FBG and 2hPG) to indicate presence of prediabetes in the participants during recruitment, especially that prediabetes subgroups exist and are associated with significantly different metabolic profiles (as highlighted in the GLARE study and narrative review). It is also equally important to measure potential changes in FBG, 2hPG and HbA1c in chronic human studies in response to interventions with plant extracts in order to determine the specific impact of intervention on the prediabetes subgroups.
5. Dietary records (three-day dietary record) may be included for chronic studies extending from the Pine Bark study and the GLARE study.
6. Future studies could also look at using mixed meals instead of liquid glucose as a better representation of a typical meal consumed.
7. As prevention strategies are increasingly emphasised in prediabetes, health practitioners may prescribe anti-diabetic drugs such as metformin for patients with prediabetes at a high risk for T2DM, especially those with BMI >35kg/m^2 or aged <60 years [158, 159]. Further research can help to answer the possibility of

combining metformin with plant extracts as a therapy for prediabetes without potential adverse effects.

8. Future studies could also look at how other effective strategies such as weight loss, increasing physical activity and improving diet quality in combination with plant extract consumption could have an additive or synergistic benefit on glycaemia in individuals with prediabetes [158-160].
9. As one of the clinical endpoints of a prediabetes clinical trial is to reduce CVD risk [161], further study may investigate how plant extracts could aid in reducing CVD risk factors such as obesity, dyslipidaemia, low high-density lipoprotein (HDL) cholesterol, high low-density lipoprotein (LDL) cholesterol, increased triglycerides (TG), increased systolic/diastolic blood pressure, enthothelial dysfunction, and inflammation (cytokines such as interleukin-6 (IL-6), C-reactive protein (CRP)) [162].
10. The measurement of changes in total and active concentrations of incretins (GLP-1) in GLARE study participants with the consumption of grape seed, rooibos tea and olive leaf extracts as another potential underlying mechanism of glucose metabolism. The measurement of GLP-1 may also ascertain the findings from the mechanistic study regarding the inhibitory action of grape seed and rooibos tea extracts on DPP4 enzyme.
11. Future studies could utilise mammalian α-glucosidase from rat intestine containing both maltase and sucrase to examine potential inhibition of extracts on α-glucosidase, another vital digestive enzyme responsible for carbohydrate metabolism [163-170]. In addition, the inhibitory action of the plant extracts, namely the New Zealand pine bark, grape seed, and rooibos tea on α-amylase could be further ascertained in hydrogen tests [171] and ^{13}C breath tests [172-174] in humans to quantify degree of delayed carbohydrate digestion in humans.
12. The other mechanistic actions as illustrated in the literature review section of the thesis could also be explored, namely the inhibition of sodium-dependent glucose co-transporter-1 (SGLT1) and sodium-independent glucose transporter-2 (GLUT2) on glucose uptake using caco-2 cell model of the human small intestine with the consumption of the plant extracts [175, 176]. A similar methodology from previous studies investigating plant extracts could be used [71, 177-179].
13. Due to the high phenolic content present in the plant extracts, these plant extracts undergo extensive metabolism in the human body [180-184]. Therefore, future

studies may investigate the metabolites generated from the consumption of the plant extracts to understand the type and extent of metabolism of the extracts and their influence on glycaemic control. This could be done by utilising an *in vitro* digestion model with the inclusion of the colonic phase, or the collection of biological samples (urine and faeces) from participants and taking measures such as polyphenol metabolite analysis and antioxidant capacity assays.

7.6 Conclusions

An early treatment in the prediabetes stage such as with plant extracts may be helpful in halting or delaying the onset of T2DM. To the best of my knowledge, this PhD research is the first to report on the glycaemic outcomes of the plant extracts: New Zealand pine bark in healthy participants, and grape seed, rooibos tea and olive leaf in the prediabetes cohort in New Zealand. The plant extracts investigated warrant further study in examining their long-term impact on glucose and insulin responses in the prediabetes cohort. In addition, a significant finding in this study was the importance of examining glycaemic responses to interventions based on the different metabolic profiles of individuals. It follows from this PhD work that treatments for prediabetes should be made available that are specific for each metabolic profile to obtain optimal glycaemic outcomes.

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Appendices

Appendix 1 – List of research outputs

Peer-reviewed journal publication
1. **Lim, W.X.J.**, Chepulis, L., von Hurst, P., Gammon, C. S., Page, R. A. *An Acute, Placebo-Controlled, Single-Blind, Crossover, Dose-Response, Exploratory Study to Assess the Effects of New Zealand Pine Bark Extract (Enzogenol (R)) on Glycaemic Responses in Healthy Participants.* Nutrients, 2020. **12**(2): p. 14.
Refereed conference/ seminar/ symposium outputs
1. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Mugridge, O., Page, R. A. *Poster presentation: The hypoglycaemic potential of antioxidant-rich plant extracts for prediabetes (GLARE study).* in *13th European Nutrition Conference, Federation of European Nutrition Societies (FENS).* 15-18 October 2019. Dublin, Ireland.
2. **Lim, W.X.J.**, Chepulis, L., von Hurst, P., Gammon, C. S., Page, R. A. *Oral presentation: A placebo-controlled, single-blind, crossover study to assess the effects of New Zealand pine bark extract (Enzogenol(R)) on glycaemic responses in healthy participants.* in *Nutrients 2019 Conference – Nutritional Advances in the Prevention and Management of Chronic Disease.* 25-27 September 2019. Barcelona, Spain.
3. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Oral presentation: A placebo-controlled, single-blind, crossover study to assess the effects of New Zealand pine bark extract (Enzogenol(R)) on glycaemic responses in healthy participants.* in *School of Health Sciences Research Seminar.* 14 August 2019. Massey University, Auckland, New Zealand.
4. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Oral presentation: A placebo-controlled, single-blind, crossover study to assess the effects of New Zealand pine bark extract (Enzogenol(R)) on glycaemic responses in healthy participants.* in *School of Sport, Exercise and Nutrition research meeting.* 7 August 2019. Massey University, Auckland, New Zealand.
5. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Poster presentation: A dose-response study to assess the effects of New Zealand pine bark extract on glycaemic responses in healthy participants.* in *Annual Meeting of the Nutrition Society of New Zealand.* 28-30 November 2018. Auckland, New Zealand.
6. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Oral presentation: Stopping the progression towards type 2 diabetes: investigating the hypoglycaemic potential of antioxidant-rich plant extracts.* in *Postgraduate and Early Career Conference.* 28 November 2018. Massey University, Auckland, New Zealand.
7. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Poster presentation: The hypoglycaemic potential of antioxidant-rich plant extracts for prediabetes.* in *Riddet Institute Student Colloquium 2018.* 10-12 July 2018. Wellington, New Zealand.
8. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Oral presentation: Stopping the progression towards type 2 diabetes: investigating the hypoglycaemic potential of antioxidant-rich plant extracts.* in *Centre of Metabolic Health Research Symposium.* 8 June 2018. Massey University, Auckland, New Zealand.

9. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Oral presentation: The hypoglycaemic potential of antioxidant-rich plant extracts*. in *Riddet Institute Student Colloquium 2017*. 5-8 November 2017. Auckland, New Zealand.
Other forms of research outputs (commercial technical reports/ visualise your thesis online video)
10. **Lim, W.X.J.**, Gammon, C. S., Page, R. A., *Technical report: Mechanistic study on the inhibitory action of Pine Bark Extract (Enzogenol(R)) on digestive enzymes (alpha-amylase and alpha-glucosidase) and DPP-4 enzyme*. 15 May 2020. p. 1-18.
11. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A. *Visualise your thesis (online): The hypoglycaemic potential of antioxidant-rich plant extracts for prediabetes*. 22 July 2019.
12. **Lim, W.X.J.**, Chepulis, L., von Hurst, P., Gammon, C. S., Page, R. A., *Technical report: The effects of rooibos tea extract on glycemic control in patients with prediabetes*. 19 July 2019. p. 1-15.
13. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A., *Technical report: Dose-response study to assess the effects of pine bark extract (Enzogenol(R)) on glycaemic response in healthy subjects. A further analysis.* 16 March 2019. p. 1-19.
14. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A., *Technical report: Dose-response study to assess the effects of pine bark extract (Enzogenol(R)) on glycaemic response in healthy subjects*. 20 November 2018. p. 1-14.
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16. **Lim, W.X.J.**, von Hurst, P., Gammon, C. S., Chepulis, L., Page, R. A., *Technical report: Summary outcome for pine bark extract study*. 23 March 2018. p. 1-8.

Appendix 2 – Pine Bark study documents

The following section of the Appendices includes participant recruitment and documents, and data collection forms pertaining to the Pine Bark study:

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Appendix 2.1 – Pine Bark study recruitment poster

RESEARCH PARTICIPANTS NEEDED

A dose-response study to assess the effects of pine bark extract (Enzogenol™) on blood sugar responses in healthy subjects

We need healthy participants **18-40 years of age!** ***Non-smoking, not taking meds, no known disease. Healthy weight. No glucose tolerance issues.***

To express interest: Contact Janice at w.x.j.lim@massey.ac.nz

All eligible participants will receive

- **$20 when screened for eligibility***
- **$60 on completion of TWO trial visits***
- **FREE breakfast for two visits**
- **FREE body composition, HbA1c value, blood lipids & blood pressure assessments**

**** Gift voucher***

Where: Massey University Nutrition Research Facility in Albany, Auckland

What is required: Must be available for 2.5h per visit to come to consume a sugary drink along with a pine bark extract capsule. Blood samples will be taken via finger pricks. You may do your own work during the visits.

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern B, Application SOA 17/73. You may contact the ethics committee: 06 356 9099 x 83657 or email humanethicsouthb@massey.ac.nz

Appendix 2.2 – Pine Bark study participant information sheet

Participant Information Sheet

Study title:	**A dose-response study to assess the effects of pine bark extract (Enzogenol™) on glycaemic (blood sugar) responses in healthy participants**		
Locality:	**Massey University Albany Campus, Auckland**	Ethics committee ref.:	SOA 17/73
Lead investigator:	**Wen Xin Janice Lim**	Contact email:	**wen.xin.lim.1@uni.massey.ac.nz**

Researcher Introduction

My name is Janice Lim, and I am a PhD student at Massey University, School of Health Sciences. My supervisors are A/Prof Rachel Page (Main), A/Prof Pam von Hurst, Dr Cheryl Gammon and Dr Lynne Chepulis.

Invitation to Participate in this Research Study

You are invited to take part in a dose-response study to assess the effects of pine bark extract (Enzogenol™) on glycaemic (blood sugar) responses in healthy participants. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 7 pages long. Please make sure you have read and understood all the pages.

WHAT IS THE PURPOSE OF THE STUDY?

Obesity and diabetes are epidemic in New Zealand (and in other Western countries), and many individuals now have problems controlling their blood sugar levels within normal levels.

Foods are ranked, nowadays, based on their effects on blood sugar levels and this is termed the glycaemic index (GI) of the food. Generally, lower GI foods are recommended for those with glucose (sugar) intolerance; however the reality is that typical New Zealand diets are high in high-GI sugars and processed foods with the average person now consuming an extra 22 teaspoons of sugar a day.

Certain foods and extracts, including berries, teas and French pine bark extracts have been shown to reduce blood sugar levels. Data suggests that this may be due to the high levels of antioxidants that are present in these foods, though other substances may also be involved. The effects of New Zealand pine bark extract on blood sugar levels has not been studied, thus this study aims to measure whether blood sugar levels can be reduced in healthy participants when taking a pine bark extract capsule along with a sugary drink.

This study is a placebo-controlled, blinded, crossover, dose-response study. A placebo-controlled study means that you will also take part in the control session where a placebo capsule will be consumed along with a sugary drink to examine the response of your blood sugar without the active ingredient. A blinded study means that you will not know what type and concentration of the capsule you will be having. A crossover study means that you, the participant, serves as your own control where you will take part in both the control visit as well as the study visit with the pine bark extract. Any difference in the results between your control and study visit can thus be attributed to the effects of pine bark extract alone. A dose-response study means that you will be having a certain concentration of the pine bark extract and your blood sugar response to it will be measured during the study.

The study is funded by NZ ENZO Nutraceuticals Ltd and the pine bark extract (Enzogenol) that will be given to you during the study sessions are provided by NZ ENZO Nutraceuticals Ltd. The pine bark extract is available for sale in NZ and approved as a food supplement in NZ and overseas. According to Regulatory Status of Enzogenol in New Zealand, Enzogenol, a Pinus radiata bark extract, is classified as a dietary supplement.

WHAT WILL MY PARTICIPATION IN THE STUDY INVOLVE?

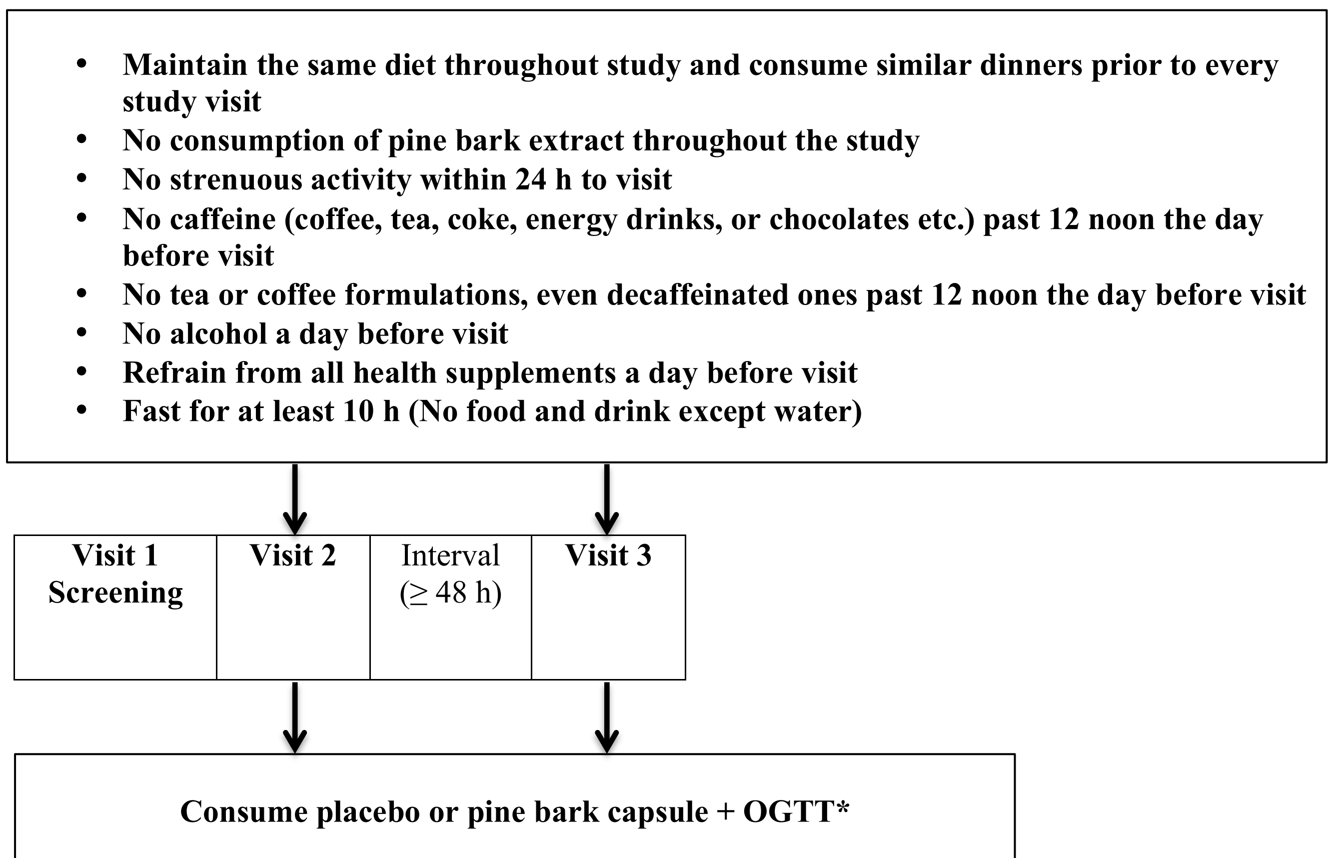
You have been chosen to participate in the study because you have met the criteria to be included in the study. The criteria includes:

- 18-40 years of age
- BMI 18.5-25.0 kg/ m^2
- HbA1c < 40 mmol/ mol
- Fasting glucose < 5.5 mmol/ L

- Not taking any medications that include blood glucose/ sugar lowering prescriptions
- Not pregnant or breastfeeding
- Not allergic to pine bark extract
- Non-smoker
- Generally healthy and not suffering from chronic diseases or diabetes
- Able to communicate well in English

The study will involve you coming to the Massey University Nutrition Research Facility, located at Albany campus, North Shore. If you are eligible after the screening visit, you will need to be available for two mornings taking approximately 2.5 hours each. Each session, including the screening visit, will require you to fast overnight (i.e. no food or drink except water) for at least 10 hours before coming to the research facility.

<u>Whole Study at a Glance</u>



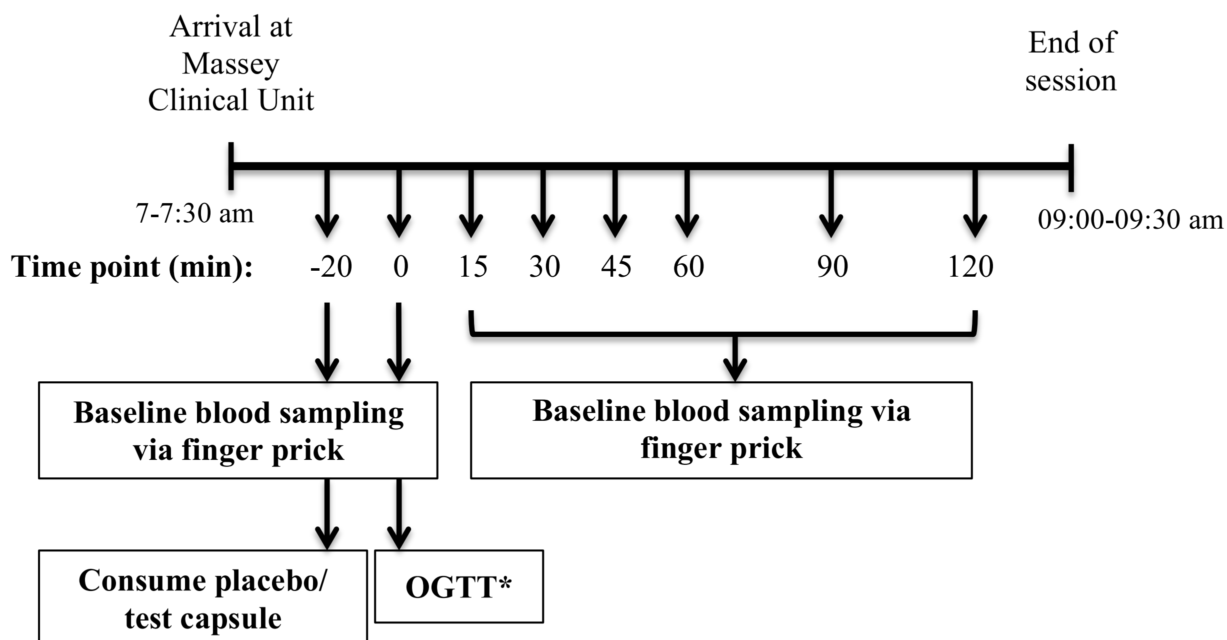
**OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*

What happens during the Screening visit (Visit 1)

During an initial screening session we will assess whether you fit these inclusion criteria. You will be required to arrive at the research facility at 7:30am ONE morning after at least a 10-hour overnight fast, to have a screening blood sample collected. The session will take approximately less than an hour. A qualified phlebotomist will measure your fasting blood sugar, lipid profile and HbA1c levels (the latter is a measure of how stable your blood sugar levels have been over the last three months) to check that you are not pre-diabetic. If your results come back and indicate that you have glucose tolerance issues you will NOT be able to participate. With your permission, we will refer you to your GP for follow-up and you may show them your blood sugar level results.

In addition, trained researchers will determine your body mass index (BMI) by measuring your weight and height. They will also be taking your waist and hip circumference, and also your percentage of body fat and lean body mass (as measured by a bioelectrical impedance scale). This can be done privately and you do not need to undress. Blood pressure and heart rate measurement will also be taken. You will also be asked about your medical history, and any medication use.

What happens during Visits 2 and 3



**OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*

During the study visits (visits 2 and 3), one or two finger-prick blood samples will be taken to measure your baseline blood sugar level. You will then be asked to consume a capsule containing 50 mg of pine bark extract, or a control capsule made of microcrystalline

cellulose. After 20 minutes you will have another baseline finger prick. You will then be asked to consume a sugary drink of 300 mL containing 75 grams of carbohydrates. You will need to remain in the research facility for the next two hours and not undertake any physical activity. Study and quiet activities are allowed, as are trips to the bathroom. Further finger prick tests will be taken at 15, 30, 45, 60, 90 and 120 minutes after consuming the sugary drink, and the blood sugar levels recorded. At the completion of this period you are welcome to eat and drink as normal (this will be provided). We will have DVDs available for viewing also to help pass the time.

You will be asked to keep your diet and lifestyle as constant as possible throughout the study and to refrain from engaging in any strenuous physical activity (walking is fine) or consumption of alcohol in the 24-hour period prior to each study session. Furthermore, avoid caffeine products (coffee, tea, coke, energy drinks, or chocolates etc.) from noon on the day prior to your study session. You will also be asked to avoid the active test foods/extracts (pine bark extract) throughout the duration of the study period (including avoiding other teas and formulations where the pine bark extract may be present).

WHAT ARE THE POSSIBLE BENEFITS AND RISKS OF THIS STUDY?

Research is a big part of medicine and healthcare and being involved can be very rewarding. You can learn a lot about the processes that are involved in research by actively participating and it can be satisfying to know that you are contributing to knowledge. Furthermore, by participating in the study you also obtain information about your general health status, HbA1c value, body composition and blood pressure measurement entirely at no cost to you. In addition, there will be a reimbursement for involvement in the study.
It is highly unlikely that you will be injured during this study. Finger prick blood sampling is safe and routinely used. A registered personnel will be available during each study session and researchers will be present to assess for adverse events (i.e. feeling nauseous, dehydrated or faint) during each blood sampling, and any event will be documented in alignment with Massey Code of Ethical Conduct. Furthermore, the pine bark extract has been tested previously and recorded as safe for consumption by the US FDA.

WHO PAYS FOR THE STUDY?

There is no cost to you, the participant, for taking part in this study.

In recognition of your time and participation in this study, you will be reimbursed $20 for screening and $30 for completion of each session. You will be reimbursed a total of $80 for completing the whole study (1 screening visit and 2 study visits) in the form of a gift voucher. If for any reason you are unable to complete the study, you will be reimbursed for your total time contributed to the study.

What if something goes wrong?

If you were injured in this study, which is unlikely, you would be eligible for compensation. The study is insured by Chubb Insurance New Zealand Limited. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

What are my rights?

Participating in this study is completely voluntary and you are free to decline to participate, decline to answer any particular question, or to withdraw from the research at any practicable time, without experiencing any disadvantage.

You, the participant has a right to access information about you, collected as part of this study. You will be told of any new information about adverse or beneficial effects related to this study which may impact upon your health.

It is important to us that we maintain your privacy throughout this study. Your name and contact information will be held electronically and stored on the Principal Investigators computer only for data recording purposes. Each participant in the study will be allocated a number. Staff involved in blood sampling and analysis will have access to participant numbers only. All data from study sessions will be recorded against your participant ID number and your name will never be used in any report, correspondence or publication. Your involvement in this study is confidential.

What happens after the study or if I change my mind?

You are able to pull out of the study at any time, and will be compensated accordingly for your time. Further you are welcome to discuss any concerns you have with the research team at any time, and you have free access to your data. If you pull out of the study all of the data that was related to you will be shredded.

The treatment intervention (pine bark extract) will not be available to any participant after the study. The study data will be stored at a secure location at Massey University Albany Campus. Electronic data and records will be the responsibility of the Principal investigator. All data will be kept for 5 years, at which point it will be destroyed using University Security methods for removal of confidential material.

Participants are welcome to discuss the findings of this study with the researchers at any time. You will also be provided with a full copy of the final study report, if requested. It is very likely that the results of this study will be written up for publication in a peer-reviewed journal and/or presentation and a Nutrition conference within 12 months of completing the study. If this happens no participant identification information will be included.

WHO DO I CONTACT FOR MORE INFORMATION OR IF I HAVE CONCERNS?

If you have any questions, concerns or complaints about the study at any stage, you can contact the following researchers involved in the study:

Dr Lynne Chepulis: Senior Research Fellow, Medical Research Unit, University of Waikato, Hamilton.
Phone: +64 (07) 3468754
Email lynne.chepulis@waikato.ac.nz

A/Prof Rachel Page: Head of School of Health Sciences
Phone 0800 627739 ext. 63462
Email: R.A.Page@massey.ac.nz

A/Prof Pam von Hurst: Associate Professor, School of Sport, Exercise and Nutrition, Massey University, Albany
Phone: +64 (09) 2136657
Email: P.R.vonHurst@massey.ac.nz

Dr Cheryl Gammon: Lecturer, School of Sport, Exercise and Nutrition, Massey University, Albany
Phone: +64 (09) 4140800 ext. 43437
Email: C.Gammon@massey.ac.nz

Wen Xin Janice Lim: PhD student, School of Health Sciences, Massey Institute Food Science & Technology, Albany
Email: wen.xin.lim.1@uni.massey.ac.nz

You may also contact the Massey University Human Ethics Committee (MUHEC) involving any concerns that you may have:

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 17/73. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz

Appendix 2.3 – Pine Bark study reference during screening

THE PINE BARK EXTRACT STUDY

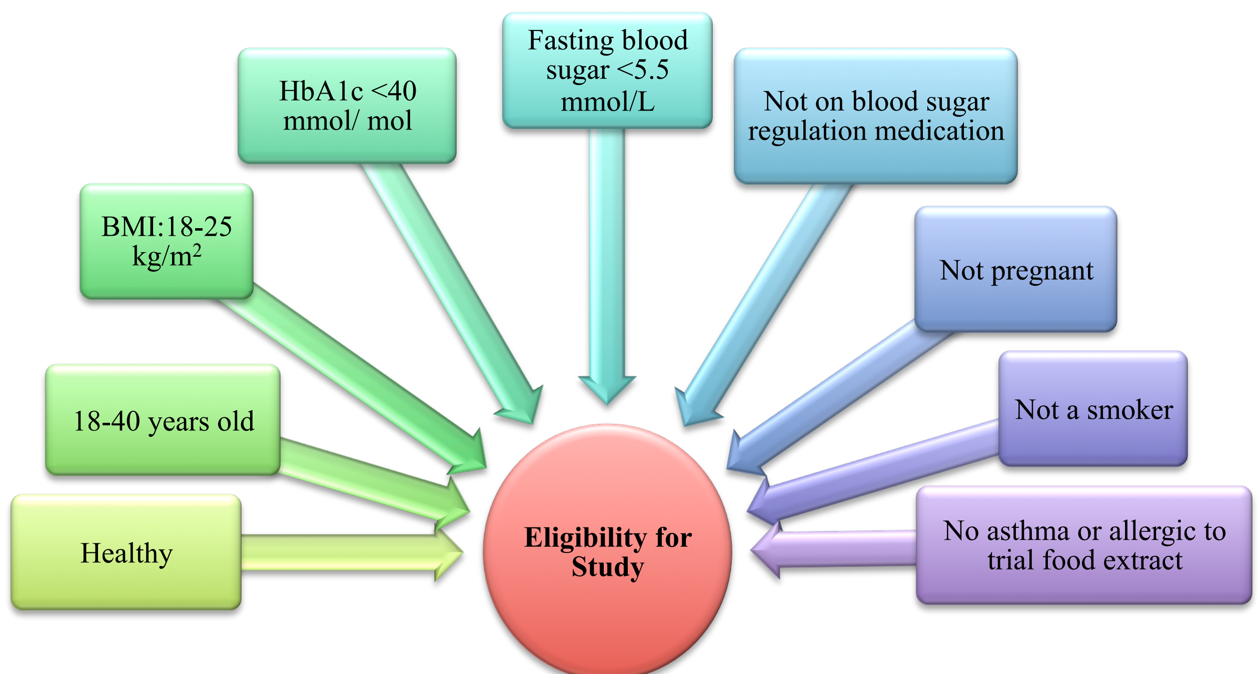
Dr. Lynne Chepulis, A/Prof Rachel Page, Dr. Pam Von Hurst, Cheryl Gammon, Owen Mugridge, Janice Lim

Study Background

Study Purpose

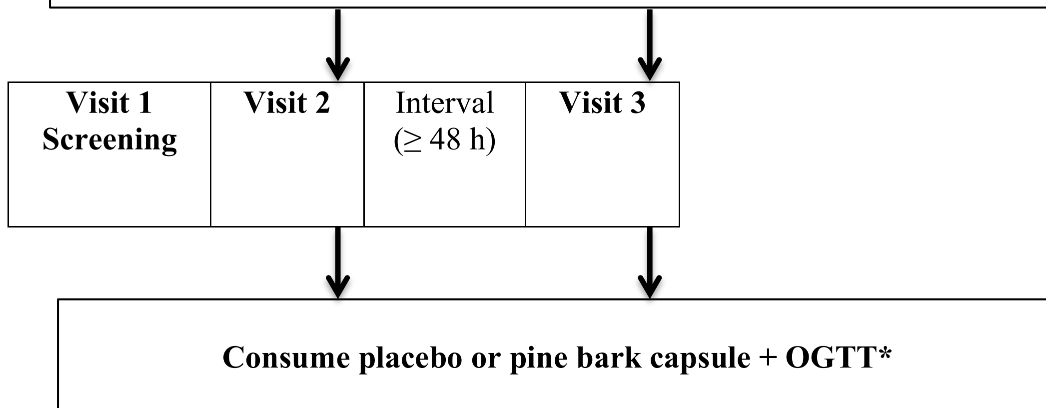
- To find out whether **natural plant extracts** can help **improve glycaemic control**
- **Prevention** better than treatment for diabetes
- The pine bark extract is provided by NZ ENZO Nutraceuticals Ltd.
- According to Regulatory status of Enzogenol, NZ pine bark (Pinus radiata) is a dietary supplement

Participation Criteria

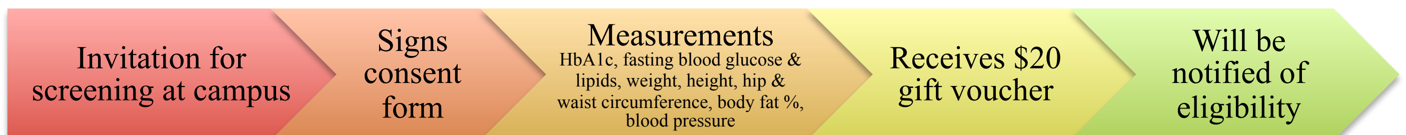


Whole Study at a Glance

- **Maintain the same diet throughout study and consume similar dinners prior to every study visit**
- **No consumption of pine bark extract throughout the study**
- **No strenuous activity within 24 h to visit**
- **No caffeine (coffee, tea, coke, energy drinks, or chocolates etc.) past 12 noon the day before visit**
- **No tea or coffee formulations, even decaffeinated ones past 12 noon the day before visit**
- **No alcohol a day before visit**
- **Refrain from all health supplements a day before visit**
- **Fast for at least 10 h (No food and drink except water)**

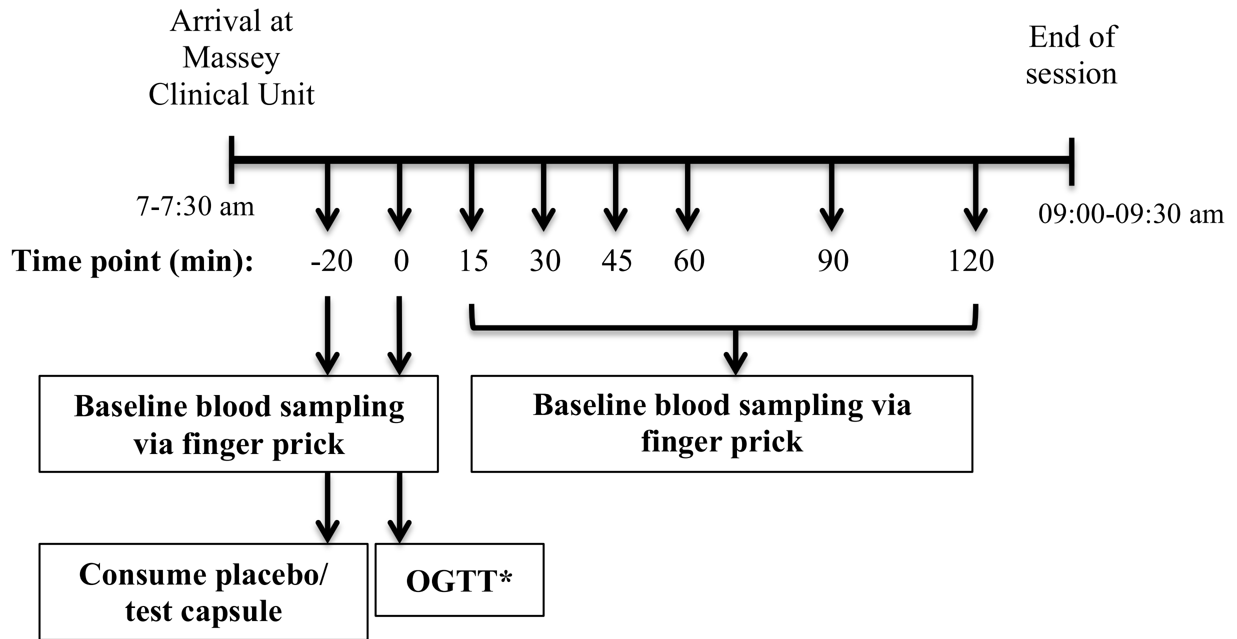


Screening Visit



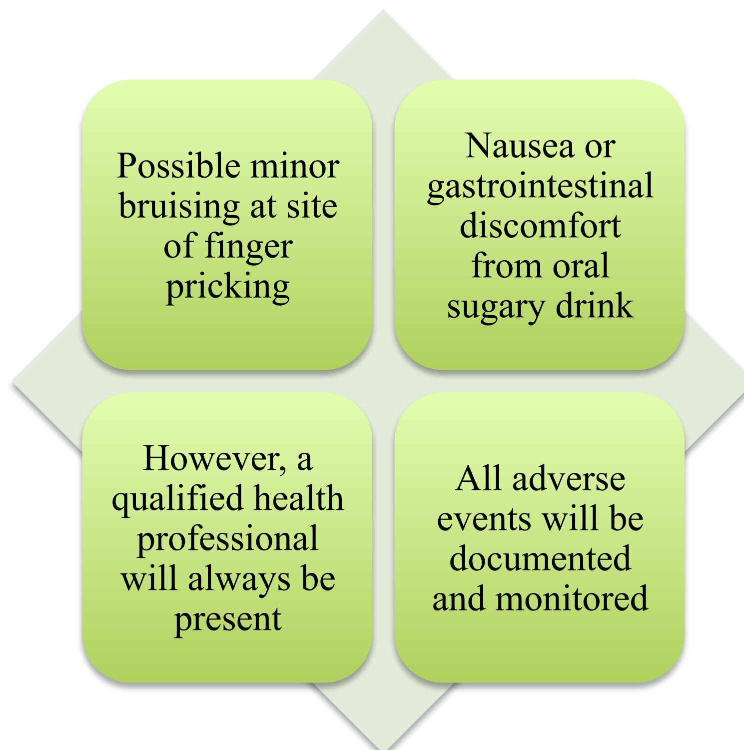
What the Study Involves

Trial Visits (2 and 3)

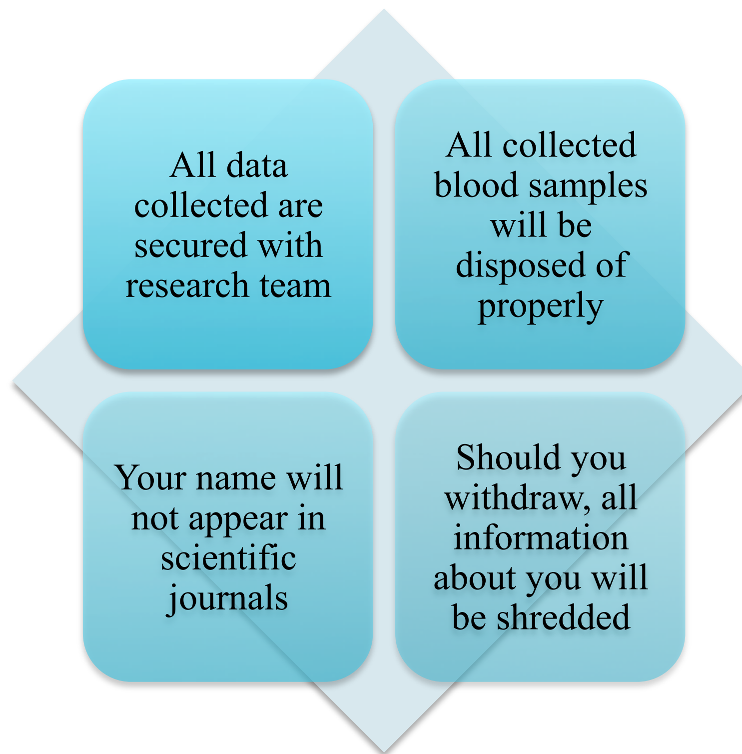


**OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*

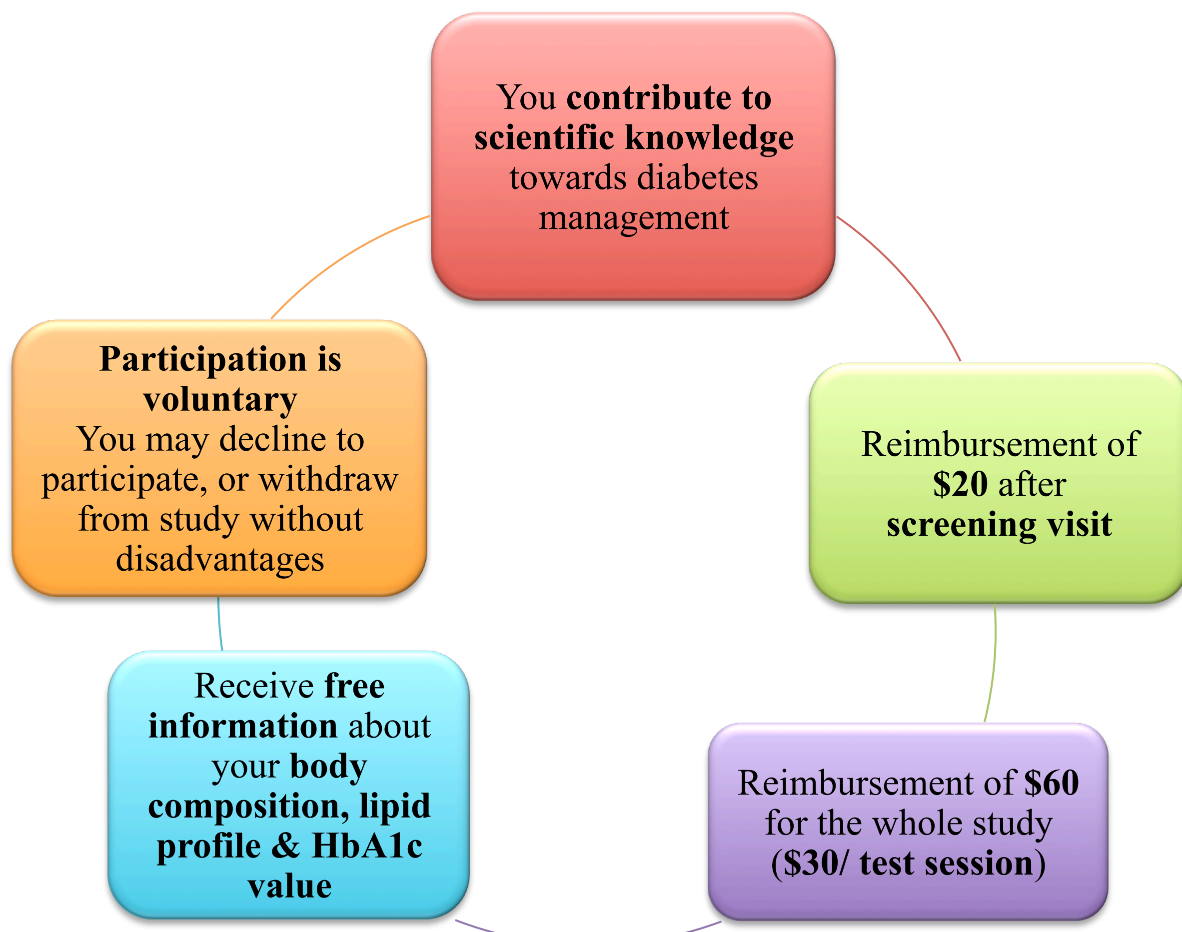
Are there any Study Risks?



Confidentiality of the Study



Your Rights and Benefits



Appendix 2.4 – Pine Bark study case record form

CASE RECORD FORM

A dose-response study to assess the effects of pine bark extract (Enzogenol™) on glycaemic (blood sugar) responses in healthy subjects

School of Health Sciences, Massey Institute of Food Science and Technology, Albany, Auckland

PRINCIPAL INVESTIGATORS:
Dr Lynne Chepulis
Assoc Prof Rachel Page
Dr Pam von Hurst
Wen Xin Janice Lim

Participant Initials: | | | |

Participant ID Number: | | | |

Date of Birth: ______________________________
DD MM YYYY

Participant ID:				Participant Initials:			

Inclusion Criteria:

1. Is the subject aged 18-40? YES / NO
2. Does the subject have a BMI between 18.5-25.0? YES / NO
3. Does the subject have an HbA1c of < 40 mmol/ mol YES / NO
4. Does the subject have a fasting glucose of < 5.5 mmol/ L YES / NO
5. Is the subject non-smoking? YES / NO
6. Is the subject able to communicate with the investigators? YES / NO

Exclusion Criteria:

1. Does the subject have any known glucose control issues / diabetes? YES / NO
2. Does the subject have any known clinically significant disease? YES / NO
3. Does the subject take any medications that include blood glucose/ sugar lowering prescriptions YES / NO
4. Does the subject have any allergies to pine bark? YES / NO
5. Pregnant or brestfeeding YES / NO

Participant ID:	☐☐☐	Participant Initials:	☐☐☐

VISIT ONE: HISTORY AND MEASUREMENTS

INFORMED CONSENT:

Written consent must be given before any study procedures can be carried out.

Has the subject given written informed consent? **YES / NO**

DEMOGRAPHIC DATA

Gender : **MALE / FEMALE**

Date of Birth ______________________________

AGE ______________________________

ETHNICITY

- NZ European
- NZ Maori
- Pacific Islander
- European
- North American
- Middle Eastern/African
- Indian
- South Asian
- Other Asian

  ____
- Other

  ____

MEASUREMENTS

Weight: ______________________ (kg)

______________________ (kg)

Height: ______________________ (cm)

______________________ (cm)

BMI: ________ (kg/m^2)

Waist: ______________________ (cm)

______________________ (cm)

Hip: ______________________ (cm)

______________________ (cm)

Waist/ Hip circumference ratio: ______________

Body fat: ____________________ (%)

Lean body mass: ______________ (%)

Participant ID: [ ][ ][ ]   Participant Initials: [ ][ ][ ]

MEDICATIONS TAKEN

Is the subject currently taking any concomitant medications/ significant non-drug therapy?

Yes [ ]   No [ ]

DETAILS:

____
____
____
____
____

KNOWN MEDICAL HISTORY

Is there any known medical history in the following systems:

	System	Yes	No			System	Yes	No
1	Cardiovascular				9	Neoplasia		
2	Respiratory				10	Neurological		
3	Hepato-billary				11	Psychological		
4	Gastro-intestinal				12	Immunological		
5	Genito-urinary				13	Dermatological		
6	Endocrine				14	Allergies		
7	Haematological				15	Eyes, ears, nose, throat		
8	Musculo-skeletal				16	Other		

If YES, please note details below:
(include any known family history of disease below)

____
____
____
____
____

Participant ID: | | | | Participant Initials: | | | |

SCREENING BLOOD TEST

Is participant fasting? YES / NO   Time of last FOOD/DRINK ______________________

Site of Collection: ______________________

Time of Collection: ______________________

Date of Collection: ______________________

FASTING GLUCOSE __________________

HbA1c ________________________

Meet Inclusion? YES / NO

Blood pressure: ____________________ (mmHg)   Heart rate: ____________________ (bpm)

Blood pressure: ____________________ (mmHg)   Heart rate: ____________________ (bpm)

Blood pressure: ____________________ (mmHg)   Heart rate: ____________________ (bpm)

Average blood pressure: _____________ (mmHg)   Average heart rate: _____________ (bpm)

Lipid values (mmol/ L):

CHO		**HDL**		Non-HDL	
TG		**LDL**		**CHOL/HDL**	

NOTES:

____

____

____

Adverse Events Schedule

The occurrence of adverse events will be sought by non-directive questioning of the subject at each visit during the study.
Adverse events also may be detected when they are volunteered by the subject during or between visits or through physical examination, laboratory test, or other assessments. As far as possible, each adverse event will be evaluated as per the below schedule.

Participant ID……………………………… Date of adverse event………………………………

Description of Adverse Event:	Comments
1. The severity (mild/ moderate/ severe)	
2. Relationship to the study intervention product (suspected/not suspected)?	
3. Duration (start and end dates or if continuing at end of study)	
4. Action taken (no action taken; intervention product permanently discontinued due to this adverse event; concomitant medication taken/ non-drug therapy given)?	

5. Whether serious/not serious, where a serious adverse event (SAE) is defined as one which is:
- ☐ Is fatal or life-threatening
- ☐ Results in persistent or significant disability/incapacity
- ☐ Constitutes a congenital anomaly/birth defect
- ☐ Requires inpatient hospitalization or prolongation of existing hospitalization, unless hospitalization is for:
- ☐ Routine treatment or monitoring of the studied indication, not associated with any deterioration in condition
- ☐ Elective or pre-planned treatment for a pre-existing condition that is unrelated to the indication under study and has not worsened since signing the informed consent
- ☐ Treatment on an emergency outpatient basis for an event not fulfilling any of the definitions of a SAE given above and not resulting in hospital admission
- ☐ Social reasons and respite care in the absence of any deterioration in the subject’s general condition
- ☐ Is medically significant, i.e., defined as an event that jeopardizes the subject or may require medical or surgical intervention to prevent one of the outcomes listed above

Additional Comments:

Research Team member ‘s name…………………………………………………

Appendix 2.5 – Pine Bark study consent form

Consent for Participation

A dose-response study to assess the effects of pine bark extract (Enzogenol™) on glycaemic (blood sugar) responses in healthy subjects

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree/ do not agree to participate as per the outlined requirements and am aware that I may withdraw at any time.

In signing this consent form I declare that I am in no way being subjected to coercion or duress and am consenting of free will.

I agree to participate in this study under the conditions set out in the Participant Information Sheet.

□ I would be willing to be added to the volunteer database for future nutrition research notifications

Signed______________________________ Date______________________

Full Name – printed____

This consent form will be kept securely according to Massey University Human Ethics Committee (MUHEC) requirements for a period of five years following the study and remain confidential throughout.

Appendix 2.6 – Pine Bark study record sheet for participants to take home

The Pine Bark Extract Study

Date:

Thank you for attending the Pine Bark Study Screening. Your results from the visit are reported below.

Measure	Result	Reference
Height (cm)		
Weight (kg)		
BMI (kg/ m2)* ** Based on NZ Heart Foundation*		< 18.5 = Underweight 18.5 - 24.9 = Normal weight 25.0 - 29.9 = Overweight > 30.0 = Obese
Waist circumference (cm)* **Based on International Diabetes Federation (IDF)*		Increased disease risk if: Men ≥ 90, while for European men ≥ 94 Women ≥ 80
Hip circumference (cm)		
Blood pressure (mm/ Hg)* ** Based on American Heart Foundation (AHA) recommendations*		See below
Heart rate (bpm)* ** Based on AHA*		60 - 100 = Normal
HbA1c (mmol/ mol)* **Based on New Zealand Medical Association (NZMA)*		≤ 40 = Normal 41-49 = Prediabetes ≥ 50 = Type 2 Diabetes
Fasting glucose (mmol/ L)* ** Based on Diabetes New Zealand*		≤ 6.0 = Normal 6.1 - 6.9 = Prediabetes ≥ 7.0 = Diabetes

Blood pressure reference:

Systolic		Diastolic	
< 120	and	< 80	Normal
120 - 129	and	< 80	Elevated
130 - 139	or	80 - 89	Stage 1 hypertension
			Stage 2 hypertension
≥ 140	or	≥ 90	Hypertension crisis

Lipid Profile*:
** Based on Southern Cross Medical Society*

Measure	Result	Recommended to be:
Total cholesterol (TC) (mmol/ L), which is HDL + LDL +20% TG		< 4.0
Triglycerides (TG) (mmol/ L)		< 1.7
High-density lipoprotein (HDL) (mmol/ L)		> 1.0
Low-density lipoprotein (LDL) (mmol/ L)		< 2.0
Non-HDL (total bad cholesterol) (mmol/ L)		< 4.0
TC/HDL ratio		< 4.0

Appendix 2.7 – Pine Bark study visit form

School of Food and Nutrition
Massey University
Albany, Auckland
New Zealand

Participant Identifier	
DOB	
Date	

The Pine Bark Extract Study

A dose-response study to assess the effects of pine bark extract (Enzogenol™) on glycaemic (blood sugar) responses in healthy participants

Form for Study Visits 2 and 3

Visit	2 3
Dietary Compliance	Yes/ No
1 day abstinence from supplements	Yes/ No
Tea or coffee abstinence	Yes/ No
Fasted at least 10-12h?	Yes/ No
Sport restriction	Yes/ No
Alcohol abstinence	Yes/ No
General condition of participant (Stress level)	High/ Medium/ Low
Name of researcher-in-charge	

Procedure	**Tick for completion**	**Time taken**	**Remarks**
Baseline blood taken at **t=-20**			
Consumption of treatment capsule			
Baseline blood taken at **t=0**			
Consumption of glucose drink			
Blood taken at **t=15**			
Blood taken at **t=30**			
Blood taken at **t=45**			
Blood taken at **t=60**			
Blood taken at **t=90**			
Blood taken at **t=120**			

Participant ID:				Participant Initials:			

THE PINE BARK STUDY DATA COLLECTION

Study visit	**Date**	**Blood glucose levels (mmol/ L) at each time point (min)**							
		-20	**0**	**15**	**30**	**45**	**60**	**90**	**120**
2									
3									

Remarks:

___

___

___

Appendix 3 – GLARE study documents

The following section of the Appendices includes participant recruitment and documents, and data collection forms pertaining to the GLARE study:

Appendix 3.1 – GLARE study recruitment poster..248
Appendix 3.2 – GLARE study online screening questionnaire..249
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Appendix 3.8 – GLARE study visit form..269

Appendix 3.1– GLARE study recruitment poster

Do you have high blood sugar?

Or know someone who is?

The School of Health Sciences at Massey University is conducting a study on the blood sugar response to antioxidant-rich plant extracts in people **diagnosed with prediabetes**, **living in Auckland.** Antioxidant-rich plant extracts may help improve blood sugar levels.

All participants will be reimbursed with:

- **$10 when screened for eligibility***
- **$200 on completion of four trial visits***
- **FREE breakfast for all visits**
- **FREE body composition, blood glucose and blood pressure assessments**

**In the form of a gift voucher*

Can you help?

To participate, you will need to make **five*** visits to the Massey University Nutrition Laboratory in Albany, Auckland. Blood samples will be taken.

**1 screening and 4 trial visits*

Grape Seed Extract

Olive Leaf Extract

Rooibos Tea Extract

This study has been approved by the Southern Health & Disabilities Ethics Committee (HDEC reference number 17/STH/82). Contact HDEC: hdecs@moh.govt.nz

To express interest for GLARE study:
www.massey.ac.nz/glarestudy
Email: glarestudy@massey.ac.nz
Phone: (09) 213 6650

To express interest for GLARE study:
www.massey.ac.nz/glarestudy
Email: glarestudy@massey.ac.nz
Phone: (09) 213 6650

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Email: glarestudy@massey.ac.nz
Phone: (09) 213 6650

Appendix 3.2 – GLARE study online screening questionnaire

Screening Questionnaires (For telephone screening):

1. **Unique ID (*Please find your ID in your email subject box)***
2. **Address**
3. **GP Name**
4. **GP Address (*Clinic, Road and Suburb of your GP)***
5. **Date of birth *(dd / mm / yyyy)***
6. **Gender**
7. **Country of Birth**
8. **Years in NZ**
9. **Which ethnic group(s) do you belong to?**
   - ✓ New Zealand European
     Maori
     Pacific
     South Asian
     Chinese
     Korean
     Southeast Asian
   - ✓ if other please specify:

10. **Whereabouts did you hear about the GLARE Study?**
    (Facebook (Massey/through a friend), word of mouth, poster, GP surgery etc.)
11. **What is your approximate height (cm)?**
12. **What is your approximate weight (kg)?**
13. **Medical History**
    Have you ever been diagnosed with any of the following:
    - ✓ Heart blood pressure
      Heart disease
      Angina
      Stroke
      Cancer
      Prediabetes
      Asthma
      None
    - ✓ if other please specify:

14. Does anyone in your immediate family (blood relative) have diabetes?

- ✓ Yes
- ✓ No
- ✓ If yes, please detail relationship, gender and age of diagnosis

15. Does anyone in your immediate family (blood relative) have cardiovascular disease?

- ✓ Yes
- ✓ No
- ✓ If yes, please detail relationship, gender and age of diagnosis

16. Does anyone in your immediate family (blood relative) have prediabetes?

- ✓ Yes
- ✓ No
- ✓ If yes, please detail relationship, gender and age of diagnosis

17. When were you diagnosed with high blood glucose/prediabetes?

18. Select here if you have not been diagnosed with high blood glucose by a doctor (Go to Q23)

- ✓ Not diagnosed

19. How long have you had high blood glucose?

20. **Date of last test (*Approximate month/year)***

21. What type of test did you have? Select all that apply.

- ✓ HbA1c
- ✓ Fasting glucose
- ✓ Don't know
- ✓ If other please specify:

22. What advice were you given in regards to having high blood glucose?

23. Have you made any lifestyle changes since being told you have high blood glucose?

24. Additional Health Questions

Please select the box if you are:

- ✓ allergic to plasters or antiseptic wipes
- ✓ have any disorder of bleeding or clotting of the blood
- ✓ have had a cold or feverish illness in the last month
- ✓ prone to fainting

- ✓ a smoker of cigarettes
- ✓ a drinker of alcohol

25. If you drink alcohol, approximately how many standard drinks per week do you drink?

26. Are you taking any prescription medications or any other medications, pills or supplements, including traditional, homeopathic medicine, or natural health ? If so, please list:

Appendix 3.3 – GLARE study participant information sheet

Participant Information Sheet

Study title: **The GLARE Study (Glucose Lowering Antioxidant Rich Plant Extracts)**

Ethics committee ref.: 17/STH/82

Locality: **Massey University Campus, Auckland**

Lead investigator: **Associate Professor Rachel Page**

Contact phone number: **0800 627739 ext. 63462**

You have been invited to take part in the GLARE (**G**lucose **L**owering **A**ntioxidant **R**ich Plant **E**xtracts) study which is looking at the effects of antioxidant-rich plant extracts on blood sugar levels in men and women. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 9 pages long, including the Consent Form. Please make sure you have read and understood all the pages.

WHAT IS THE PURPOSE OF THE STUDY?

The purpose of the study is to look at certain antioxidant-rich plant extracts (which include grape seed, rooibos tea and olive leaf extract) and the impact they have on blood sugar levels in people diagnosed with prediabetes. Prediabetes is a warning sign for development of type 2 diabetes. It is a term given for when you have blood sugar levels higher than normal but not high enough yet to be considered type 2 diabetes. Antioxidants are substances that are able to fight against unstable molecules formed in the body. The plant extracts chosen for this study contain plenty of antioxidants.

This study aims to build on information from a recent study (Chepulis et al, 2016), which showed that 4 antioxidant-rich plant extracts (amla berry, grape seed, rooibos tea and green tea extracts) had a positive impact on blood sugar response in subjects with normal blood sugar levels. Little is known about whether these plant extracts can be used to improve blood sugar control in people with prediabetes, or whether they can slow the development to type 2 diabetes (a more serious condition than prediabetes).This study aims to examine the short term effects of these antioxidant-rich plant extracts (rooibos tea, grape seed and olive leaf) on blood sugar control in people with prediabetes.

The sources of funding for this study have been provided by the Massey University Research Fund, COMVITA NZ Ltd., Rooibos Council of South Africa and Toiohomai Institute Technology. Should you have any questions about this study during your participation in the study you can contact the following people:

***Dr Lynne Chepulis:** Senior Research Fellow, Medical Research Unit, University of Waikato, Hamilton*

Phone +64 7 837 9553 *(work) or 022 675 3353*
Email: lynne.chepulis@waikato.ac.nz

***A/Prof Rachel Page:** Head of School of Health Sciences, Massey University*
Phone 0800 627739 ext. 63462
Email: R.A.Page@massey.ac.nz

***Dr. Pam Von Hurst** : Associate Professor, School of Sport, Exercise and Nutrition, Massey University, Albany Campus*
Phone: +64 (09) 2136657
Email: P.R.vonHurst@massey.ac.nz

The ethical considerations of this study have been approval by the Southern Heath & Disabilities Ethics Committee (HDEC). You can contact HDEC via email on hdecs@moh.govt.nz

WHAT WILL MY PARTICIPATION IN THE STUDY INVOLVE?

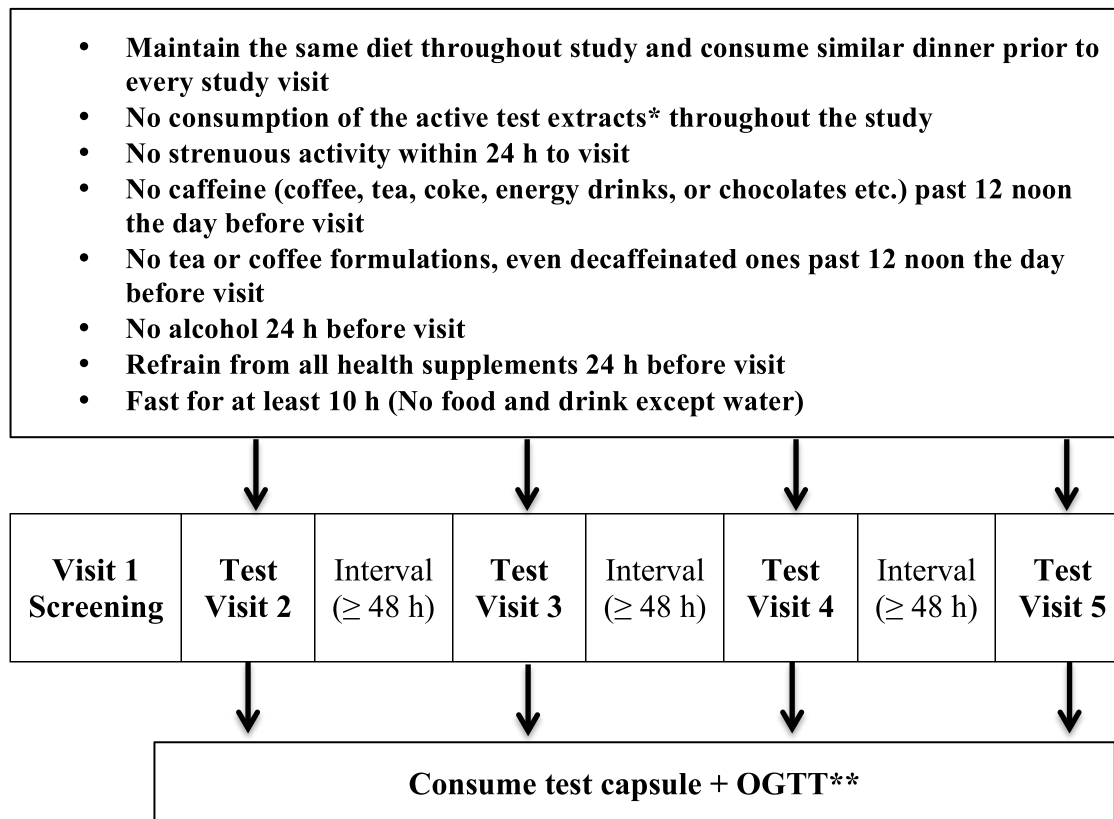
You have been chosen to participate in the study because you have met the criteria to be included in the study. The criteria includes:

- *A high blood sugar level (known as prediabetes), (HbA1c between 41-49 mmol/mol)*
- *Not being on medication that influences blood sugar levels*
- *Not a smoker*
- *Able to speak and read English*

*The study will involve you coming to the Massey University Food & Nutrition Laboratory, located at the Albany Campus, North Shore. You will need to be available for **FIVE** visits at your preferred time. Each visit will be for approximately 2.5 hours from 7 -7.30 am – 9.30 - 10 am in the morning. You will be required to fast overnight (i.e. no food or drink except*

water) for at least 10 hours prior to coming into the Lab for each visit (with the exception of your first screening visit). The research team will provide you with breakfast and tea or coffee after each of your visits.

<u>The whole study at a glance</u>



**Active test extracts are the grape seed, rooibos tea and olive leaf extracts*

***OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*

*Your **first** visit will be the screening visit, which will include collecting the following data:*

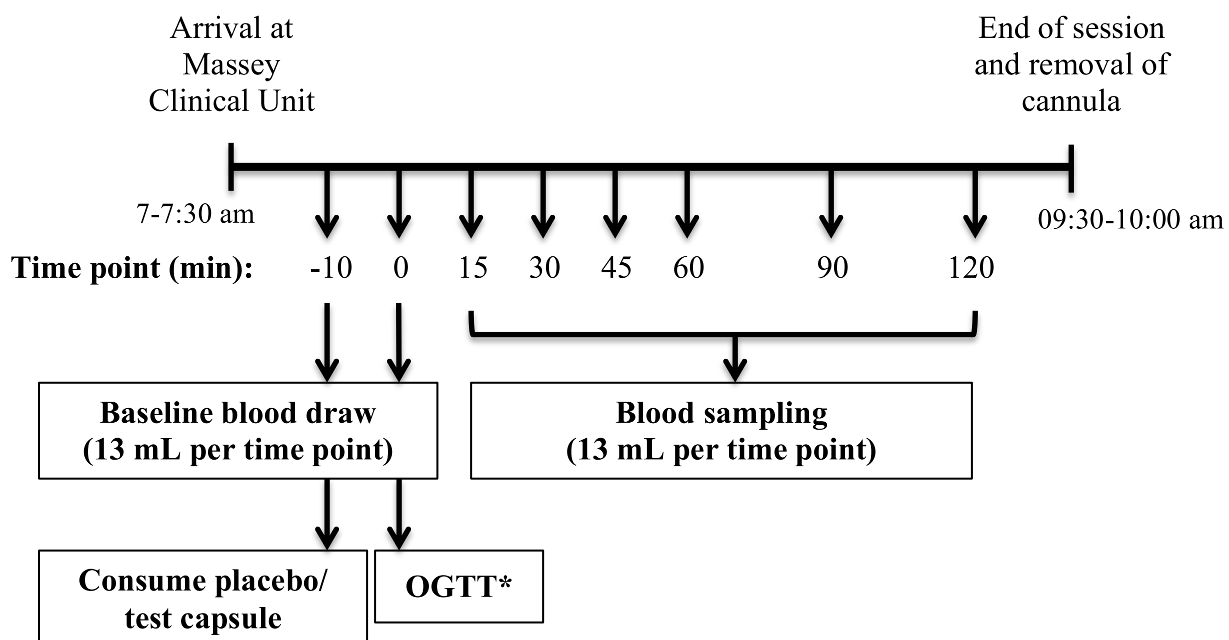
- *Collection of some personal health information such as weight, height, hip and waist circumference, body fat percentage (as measured by a bioelectrical impedance (BIA) scale), blood pressure, medical history, and any medication use.*
- *Additional information will be collected around your diagnosis of prediabetes, particularly around duration of prediabetes and the dietary and lifestyle advice given to you at the time of diagnosis.*

*Your **subsequent** visits (visits 2-5) will involve consuming either a placebo or antioxidant-rich plant extract capsule and drinking a 300 mL sugary drink (contains 75 g of glucose). This study is a placebo-controlled blinded study. This means that you will not know what test sample i.e. antioxidant-rich plant extract in a capsule or control (placebo capsule) you will*

be consuming with a sugary drink. The placebo capsule contains no antioxidant or active substance and acts as a control as it will have no effect on your blood sugar levels.

*The **last visit** will also involve collecting personal health information again (including; weight, waist and hip circumference, body fat percentage and blood pressure). Blood pressure will be recorded at every visit.*

<u>What happens at Visits 2-5</u>



**OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*
All blood samples collected will be used to quantify blood glucose levels, insulin levels, antioxidant capacity, and peptide levels.

When you arrive at 7-7.30 am on your visit, a small plastic chute will be inserted into one of your veins in your arm to allow a blood sample to be taken to measure your baseline blood sugar level. Ten minutes before drinking a 300 mL sugary drink you will be given a capsule that will either contain an antioxidant-rich plant extract (rooibos, olive leaf or grape seed extract) or a placebo capsule. Once you have consumed your sugary drink, you will then need to remain in the Massey University Food and Nutrition lab for the next two and half hours and not undertake any physical activity. Study and quiet activities are allowed, as are trips to the bathroom. Further blood samples will be taken from the same chute at 15, 30, 45, 60, 90 and 120 minutes after consuming the antioxidant-rich extract or placebo, and the blood sugar levels, insulin levels and antioxidant activity will be determined at a later date. We will have qualified phlebotomists (people trained to draw blood) on site to collect these blood samples.

At the completion of your 2.5 hour visit you are welcome to eat and drink as normal (this will be provided). We will have DVDs available for viewing also to help pass the time.

What happens between Visits 2-5

You will be asked to keep your diet and lifestyle as constant as possible throughout the study and to refrain from engaging in any strenuous physical activity (walking is fine) or consumption of alcohol in the 24-hour period prior to each test session. You will also be asked to avoid the active test foods/extracts for at least seven days prior to the first test session and throughout the duration of the test period, including avoiding other teas and formulations where the extracts may be present.

The research team understands that some of the information that we are collecting is very sensitive and it will be treated as such. Only the research team and you, the participant, will have knowledge of your personal information.

WHAT ARE THE POSSIBLE BENEFITS AND RISKS OF THIS STUDY?

Direct benefits of participating in this study include; an increased awareness and knowledge of the processes involved in research by actively participating in it, and a satisfaction in knowing that you are contributing to nutrition knowledge in the community. Additionally, there is also a monetary payment for involvement in this study.

Foreseeable risks, adverse-effects and discomforts that you may encounter by taking part in this study are minimal, but could include possible infection from the site in which blood is drawn and there may be some minor bruising at this site as well. You may also feel some nausea or gastrointestinal discomfort from ingestion of the glucose (in the sugary drink) for the oral glucose tolerance test. These discomforts will be managed by the presence of a qualified health professional who will be available to assist you should you require it. A record of all adverse events will be monitored and maintained throughout the course of the study.

WHO PAYS FOR THE STUDY?

There is no cost to you, the participant, for taking part in this study.

In recognition for your time and participation in this study, you will be reimbursed a total of $210. This total amount is based on your attendance to all FIVE visits to the Massey University Food and Nutrition Laboratory on Albany campus. If for any reason you are unable to complete the study, you will be reimbursed for your total time contributed to the study. You will receive $10 for completing the screening visit and then for each study visit that you complete, you will be reimbursed with $50.

WHAT IF SOMETHING GOES WRONG?

If you were injured in this study, which is unlikely, you would be eligible for compensation from ACC just as you would be if you were injured in an accident at work or at home. You will have to lodge a claim with ACC, which may take some time to assess. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

WHAT ARE MY RIGHTS?

Participating in this study is completely voluntary and you are free to decline to participate, or to withdraw from the research at any practicable time, without experiencing any disadvantage.

You, the participant has a right to access information about you, collected as part of this study.

You will be told of any new information about adverse or beneficial effects related to this study which may impact upon your health.

It is important to us that we maintain your privacy throughout this study. Your name and contact information will be held electronically and stored on the Principal Investigators computer only for data recording purposes. Each participant in the study will be allocated a number. Staff involved in blood sampling and analysis will have access to participant numbers only. All data from test sessions will be recorded against your participant ID number and your name will never be used in any report, correspondence or publication. Your involvement in this study is confidential.

WHAT HAPPENS AFTER THE STUDY OR IF I CHANGE MY MIND?

You are able to pull out of the study at any time, and will be compensated accordingly for your time. Further you are welcome to discuss any concerns you have with the research team at any time, and you have free access to your data. If you pull out of the study all of the data that was related to you will be shredded.

The treatment intervention (antioxidant capsules being tested in the GLARE study) will not be available to any participant after the study. The outcomes of this study will enable selection of antioxidant-rich plant extracts that can be investigated further in a long term intervention study.

The study data will be stored at a secure location at Massey University Albany Campus. Electronic data and records will be the responsibility of the Principal investigator. All data will be kept for 10 years, at which point it will be destroyed using University Security methods for removal of confidential material. At the completion of the study all biological samples collected will be disposed of using established methods for discarding biological waste. Any participant can request to have their remaining blood sample returned to them.

You may hold beliefs about a sacred and shared value of all or any tissue samples removed. The cultural issues associated with sending your samples overseas and/or storing your tissue should be discussed with your family/whanau as appropriate. There are a range of views held

by Maori around these issues; some iwi disagree with storage of samples citing whakapapa and advise their people to consult prior to participation in research where this occurs. However it is acknowledged that individuals have the right to choose.

We anticipate that the results of this study will be published in a peer-reviewed journal within 12 months of completing the study. Participants are welcome to discuss the findings of this study with the researchers at any time.

WHO DO I CONTACT FOR MORE INFORMATION OR IF I HAVE CONCERNS?

If you have any questions, concerns or complaints about the study at any stage, you can contact:

Researchers in the Study:

You can contact the following researchers: A/Prof Rachel Page, Dr Lynne Chepulis or Dr Pam von Hurst. Contact details are at the beginning of this information sheet.

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

Phone: 0800 4 ETHICS
Email: hdecs@moh.govt.nz

Consent Form

Please tick to indicate you consent to the following *(Add or delete as appropriate)*

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	Yes ☐	No ☐
I have been given sufficient time to consider whether or not to participate in this study.	Yes ☐	No ☐
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	Yes ☐	No ☐
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	Yes ☐	No ☐
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	Yes ☐	No ☐
I consent to the research staff collecting and processing my information, including information about my health.	Yes ☐	No ☐
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes ☐	No ☐
I consent to my GP or current provider being informed about my participation in the study and of any significant abnormal results obtained during the study.	Yes ☐	No ☐
I am aware that my blood samples will be disposed of using established guidelines for discarding biological waste.	Yes ☐	No ☐
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	Yes ☐	No ☐
I understand the compensation provisions in case of injury during the study.	Yes ☐	No ☐
I know who to contact if I have any questions about the study in general.	Yes ☐	No ☐
I understand my responsibilities as a study participant.	Yes ☐	No ☐
I wish to receive a summary of the results from the study.	Yes ☐	No ☐

Declaration by participant:
I hereby consent to take part in this study.

Participant's name: ______________________________

Signature: ______________________ Date: ______________________

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name: ______________________________

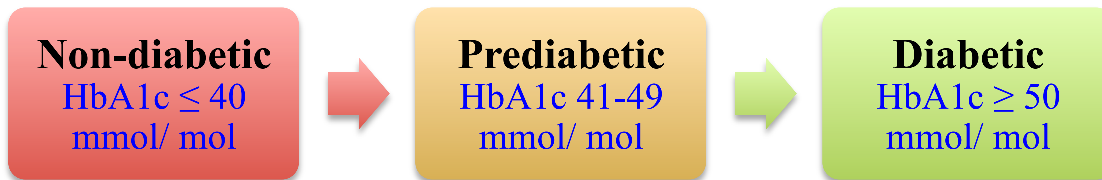
Signature: ______________________ Date: ______________________

Appendix 3.4 – GLARE study reference during screening

THE GLARE STUDY

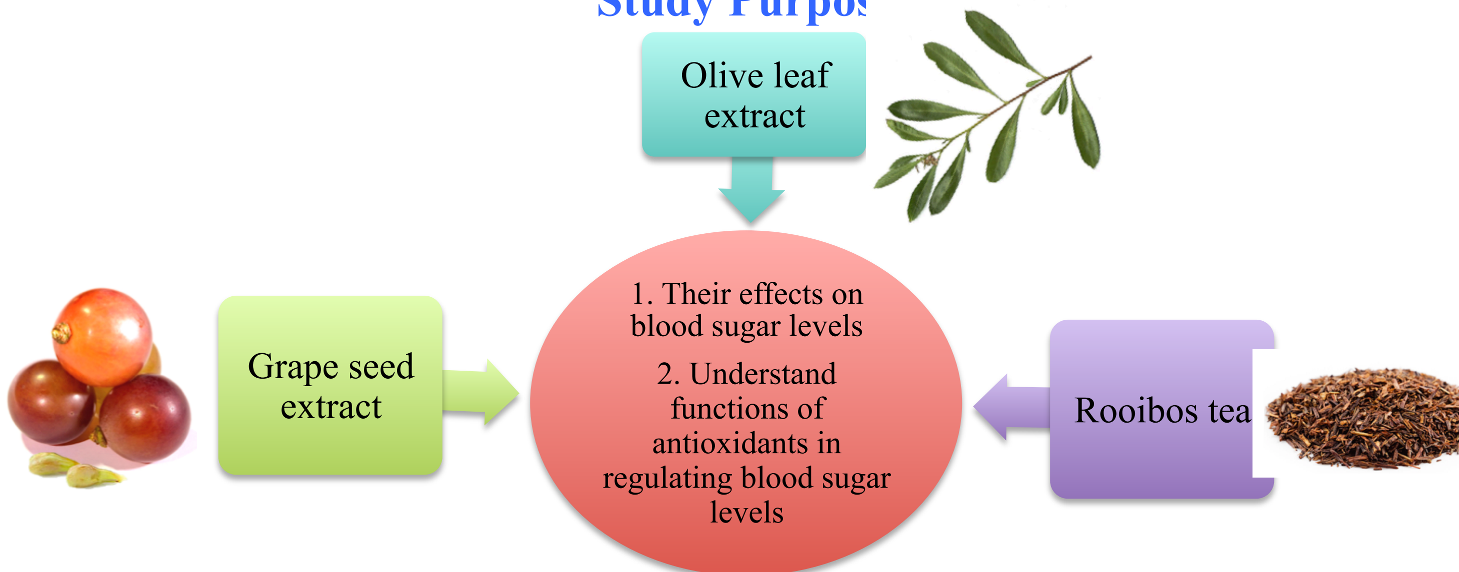
Research team: Dr. Lynne Chepulis, A/Prof Rachel Page, Dr. Pam Von Hurst, Cheryl Gammon, Owen Mugridge, Alexandra Tava, Jasmine Foote, Janice Lim

Study Background

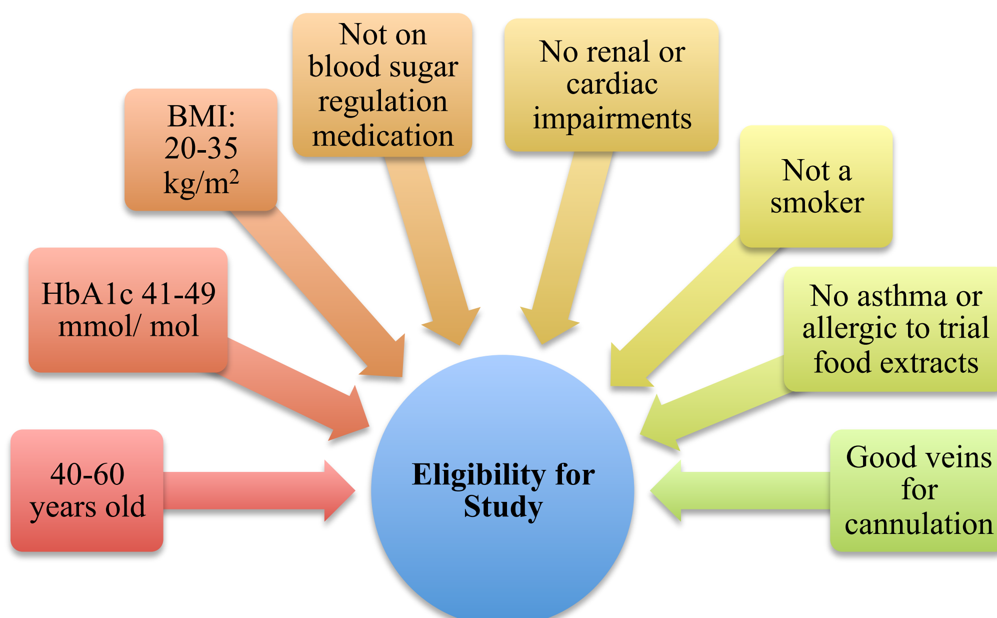


- **Prediabetes** is where you have blood sugar levels above normal but below defined thresholds of diabetes
- **High risk** for diabetes with **5-10%** conversion rate every year
- **Goal** to achieve **normal blood sugar levels** in prediabetic people
- **Prevention** better than treatment for diabetes
- **Natural food sources** may be a **better strategy** than diabetic medication

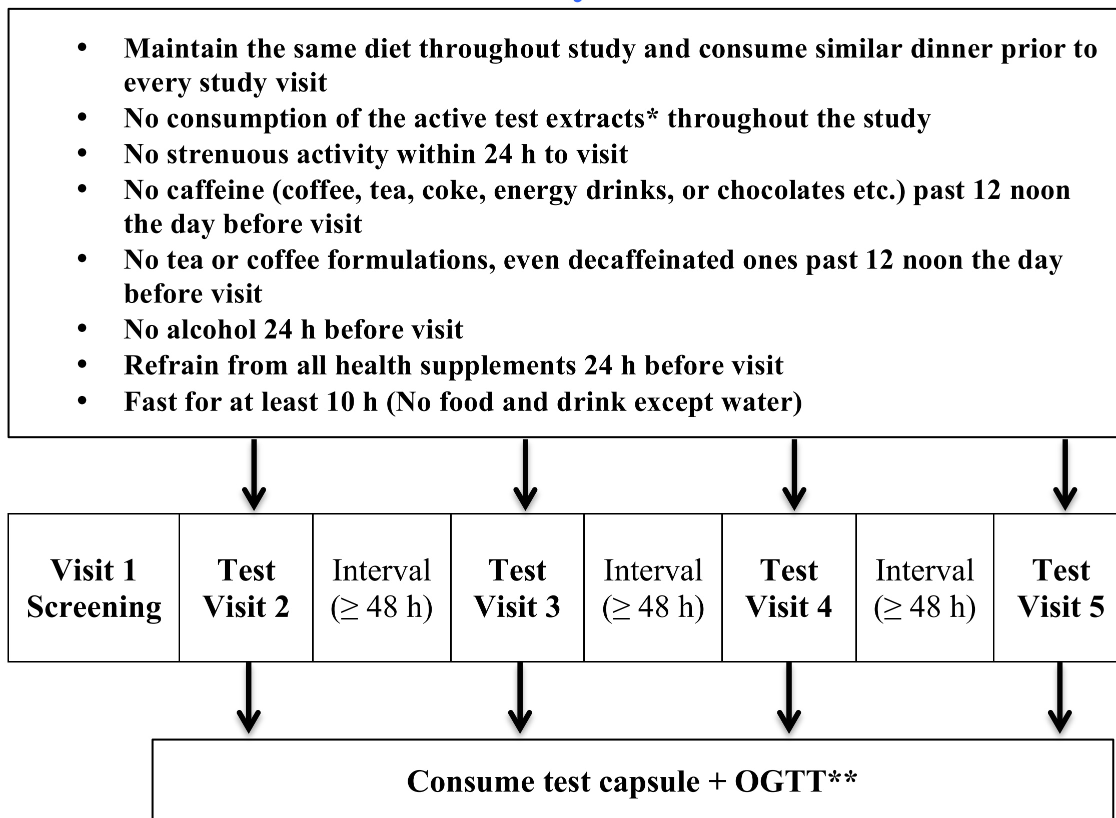
Study Purpose



Participation Criteria



Whole Study at a Glance

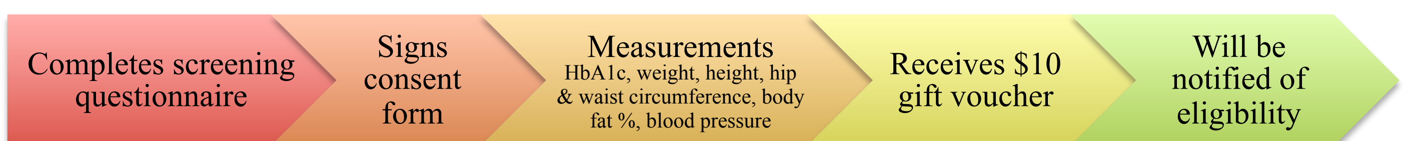


**Active test extracts are the grape seed, rooibos tea and olive leaf extracts*

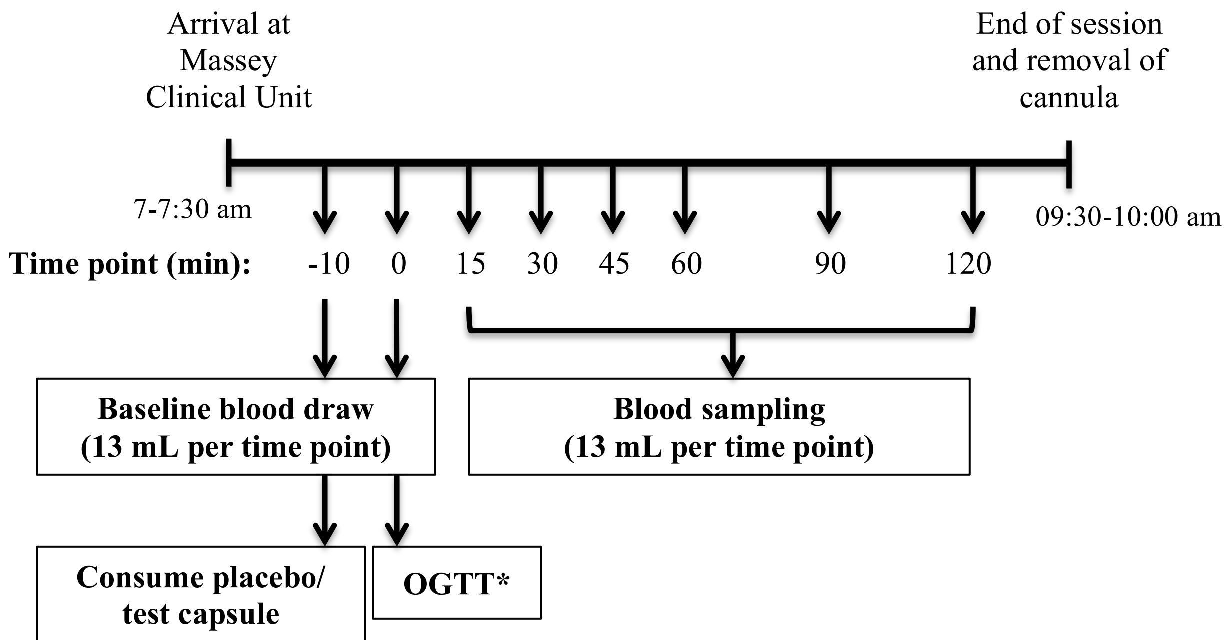
***OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*

What the Study Involves

Screening Visit

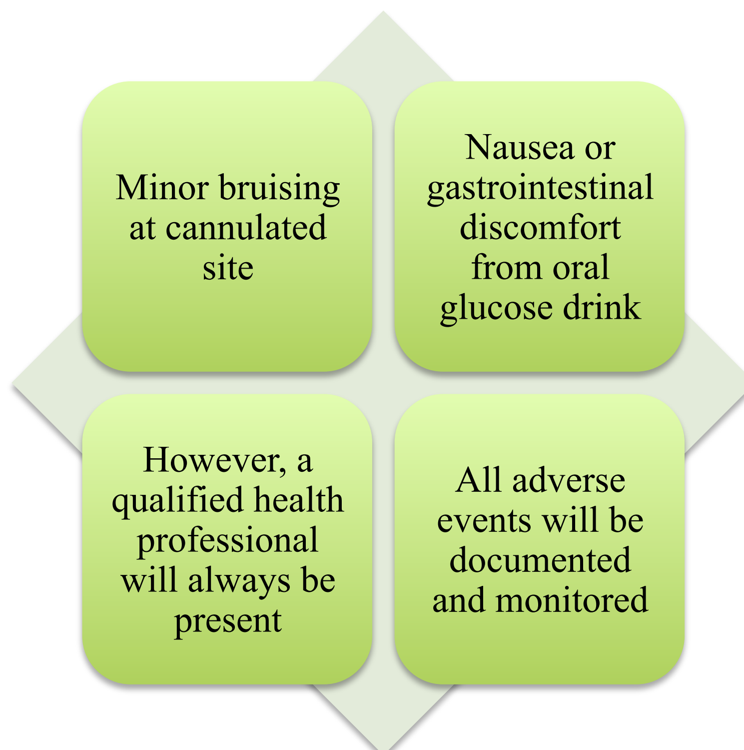


Trial Visits (2-5)

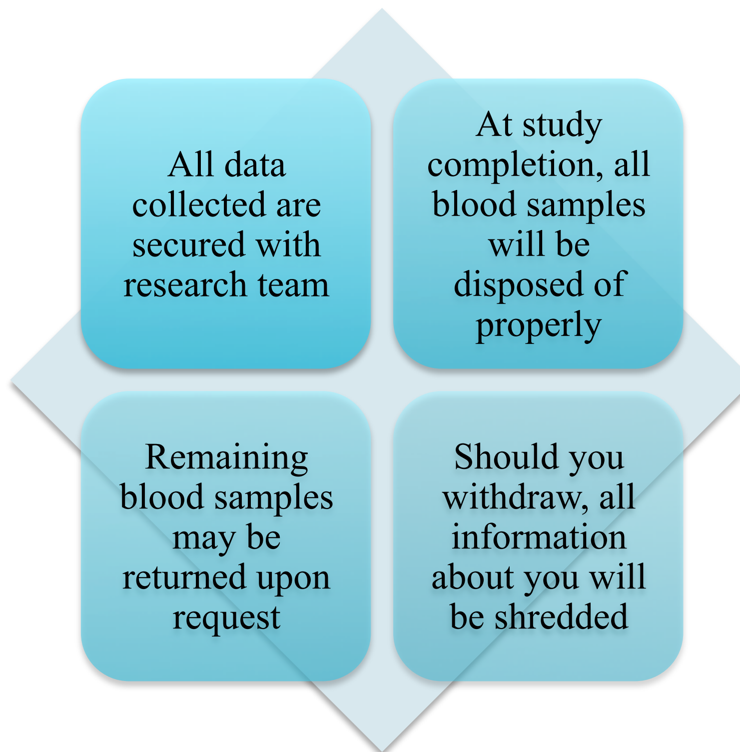


**OGTT stands for Oral Glucose Tolerance Test, which comprises a 300 mL sugary drink (containing 75 g carbohydrates).*
All blood samples collected will be used to quantify blood glucose levels, insulin levels, antioxidant capacity, and peptide levels.

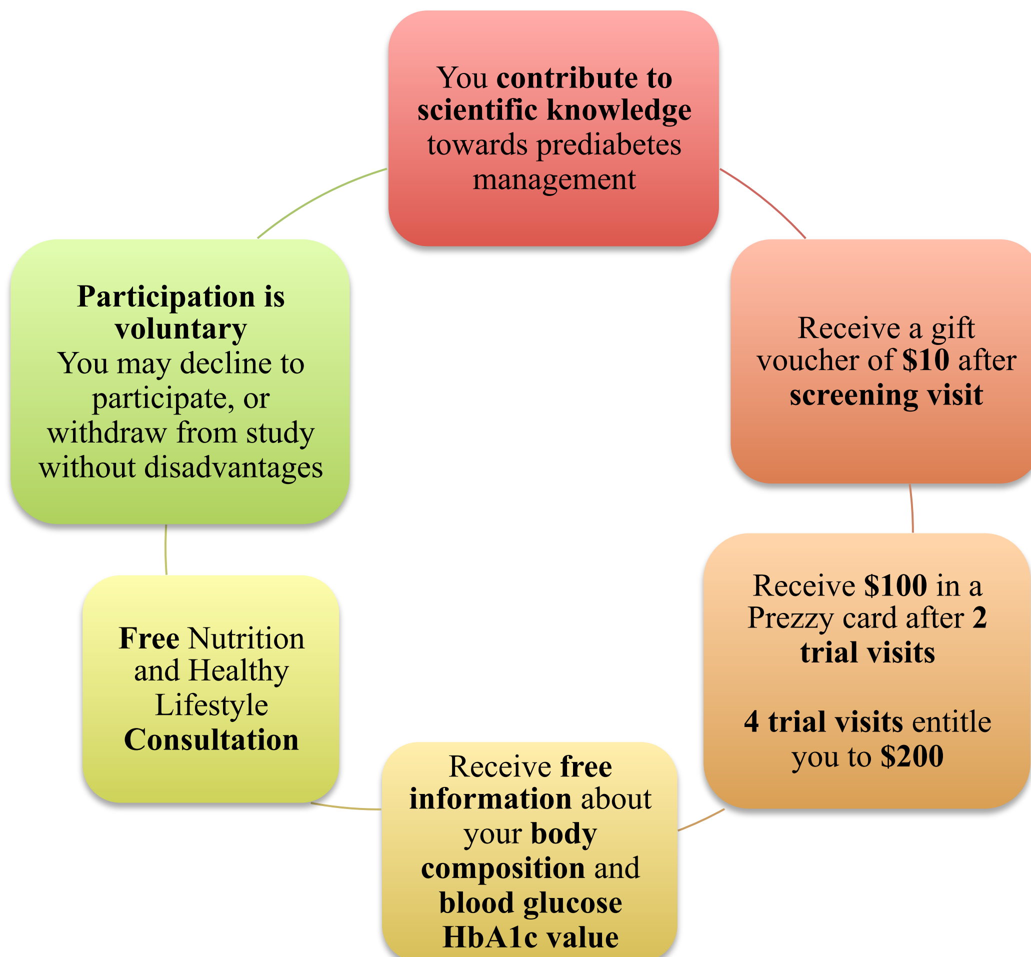
Are there any Study Risks?



Confidentiality of the Study



Your Rights and Benefits



Appendix 3.5 – GLARE study case record form

GLARE Study
Date:

ID		**DOB**	
Consent			
Screening check		> duration of prediabetes > dietary and lifestyle advice given to you at diagnosis	
Medications/ supplements			
Height (cm)			
Weight (kg)			
BMI			
BIA BF%			
Waist circumference (cm)			
Hip circumference (cm)			
Blood Pressure	/	/	/
Heart Rate (bpm)			
HbA1c			
If F - Are you post-menopausal? (Answer YES, if your last menstrual period was over one year ago?) Y/N			
Notes eg. Veins/ blood draw experience.			
Voucher			

Adverse Events Schedule

The occurrence of adverse events will be sought by non-directive questioning of the subject at each visit during the study.
Adverse events also may be detected when they are volunteered by the subject during or between visits or through physical examination, laboratory test, or other assessments. As far as possible, each adverse event will be evaluated as per the below schedule.

Participant ID………………………………   Date of adverse event……………………………….

Description of Adverse Event:	Comments
1. The severity (mild/ moderate/ severe)	
2. Relationship to the study intervention product (suspected/not suspected)?	
3. Duration (start and end dates or if continuing at end of study)	
4. Action taken (no action taken; intervention product permanently discontinued due to this adverse event; concomitant medication taken/ non-drug therapy given)?	

5. Whether serious/not serious, where a serious adverse event (SAE) is defined as one which is:
- ☐ Is fatal or life-threatening
- ☐ Results in persistent or significant disability/incapacity
- ☐ Constitutes a congenital anomaly/birth defect
- ☐ Requires inpatient hospitalization or prolongation of existing hospitalization, unless hospitalization is for:
- ☐ Routine treatment or monitoring of the studied indication, not associated with any deterioration in condition
- ☐ Elective or pre-planned treatment for a pre-existing condition that is unrelated to the indication under study and has not worsened since signing the informed consent
- ☐ Treatment on an emergency outpatient basis for an event not fulfilling any of the definitions of a SAE given above and not resulting in hospital admission
- ☐ Social reasons and respite care in the absence of any deterioration in the subject's general condition
- ☐ Is medically significant, i.e., defined as an event that jeopardizes the subject or may require medical or surgical intervention to prevent one of the outcomes listed above

Additional Comments:

Research Team member 's name…………………………………………………

Appendix 3.6 – GLARE study consent form

Consent for Participation in the GLARE Study

This form should only be completed and signed if there is full agreement with the details and terms given below.

I ________________________________ (full name) give my full consent to participate in the study "A placebo-controlled, single blind trial to assess the effect of antioxidant-rich plant extracts on glucose levels in prediabetic subjects" as conducted by principal investigator Dr. Lynne Chepulis and the GLARE team.

I have read the information sheet provided to me that details the study, my role and responsibilities and the role and responsibilities of the research team.

I have had the opportunity to discuss the study with the researchers and to ask questions and have received a satisfactory response for any concerns I have voiced.

I agree to participate as per the outlined requirements and am aware that I may withdraw at any time.

In signing this consent form I declare that I am in no way being subjected to coercion or duress and am consenting of free will.

I understand that involvement in this study requires me to undertake a number of oral glucose tolerance tests, but that this will be done under the supervision of a study team member.

☐ I would be willing to be added to a volunteer database for future nutrition research notifications

Signed____________________________ Date____________________

This consent form will be kept securely according to the Health & Disability Ethics Committee requirements for a period of seven years following the study and remain confidential throughout.

Appendix 3.7 – GLARE study record sheet for participants to take home

The GLARE Study

Date:

Thank you for attending the GLARE Study Screening. Your results from the visit are reported below.

Measure	Result	Reference
Height (cm)		
Weight (kg)		
BMI (kg/ m2)* ** Based on NZ Heart Foundation*		< 18.5 = Underweight 18.5 - 24.9 = Normal weight 25.0 - 29.9 = Overweight > 30.0 = Obese
Waist circumference (cm)* **Based on International Diabetes Federation (IDF)*		Increased disease risk if: Men ≥ 90, while for European men ≥ 94 Women ≥ 80
Hip circumference (cm)		
Blood pressure (mm/ Hg)* ** Based on American Heart Foundation (AHA) recommendations*		See table below
Heart rate (bpm)* ** Based on AHA*		60 - 100 = Normal
HbA1c (mmol/ mol)* **Based on New Zealand Medical Association (NZMA)*		≤ 40 = Normal 41-49 = Prediabetes ≥ 50 = Type 2 Diabetes
Notes		

Blood pressure reference:

Systolic		Diastolic	
< 120	and	< 80	Normal
120 - 129	and	< 80	Elevated
130 - 139	or	80 - 89	Stage 1 hypertension
≥ 140	or	≥ 90	Stage 2 hypertension
> 180	and/ or	> 120	Hypertension crisis

Appendix 3.8 – GLARE study visit form

School of Food and Nutrition
Massey University
Albany, Auckland
New Zealand

Participant Identifier	
DOB	
Date	

GLARE Study

A placebo-controlled blinded trial to investigate the effect of antioxidant-rich food extracts on postprandial blood glucose response in people with prediabetes.

Form for Study Visits 2-5

Visit	2 3 4 5
Dietary Compliance	Yes/ No
1 day abstinence from supplements	Yes/ No
Fasted at least 10-12h?	Yes/ No
Abstinence from tea coffee since 12nn?	Yes/ No
Sport restriction?	Yes/ No
Alcohol abstinence?	Yes/ No
Taking medication (*if any*) consistently?	Yes/ No
General condition of participant (Stress level)	High/ Medium/ Low
Intervention	C E1 E2 E3
Blood pressure (mm Hg):	
Heart rate (bpm):	
Name of researcher-in-charge	

Procedure	Tick for completion	Actual time	Remarks
Baseline blood taken at t=-10			
Consumption of treatment capsule			
Baseline blood taken at t=0			
Consumption of glucose drink			
Blood taken at t=15			
Blood taken at t=30			
Blood taken at t=45			
Blood taken at t=60			
Blood taken at t=90			
Blood taken at t=120			

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

School of Food and Nutrition
Massey University
Albany, Auckland
New Zealand

Participant Identifier	
DOB	
Date	

GLARE Study

A placebo-controlled blinded trial to investigate the effect of antioxidant-rich food extracts on postprandial blood glucose response in people with prediabetes.

Form for Laboratory Processing of Blood Sample

Visit	2 3 4 5
Dietary Compliance	Yes/ No
1 day abstinence from supplements	Yes/ No
Fasted at least 10-12h	Yes/ No
Abstinence from tea coffee since 12nn?	Yes/ No
Sport restriction?	Yes/ No
Alcohol abstinence?	Yes/ No
Taking medication (*if any*) consistently?	Yes/ No
General condition of participant (Stress level)	High/ Medium/ Low
Intervention	C E1 E2 E3
Treatment	Placebo/ Grape seed/ Rooibos tea/ OLE
Name of researcher-in-charge	

Blood Sample Processing

Time	**Actual start time**	**Procedure**	**Tick for completion**	**Remarks**
		Blood put in ice box at **t=-10** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	**ORAC test to be done*
		Blood put in ice box at **t=0** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	

		Blood put in ice box at **t=15** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	***Red serum tube (S10, t=-10) to be centrifuged.**
		Blood put in ice box at **t=30** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	***Red serum tube (t=0) to be centrifuged.**
		At t=30 for Incretins (0 & 30): - *Centrifuge EDTA tubes at 2900 rpm for 10min at 4°C* - *Aliquot samples for storage at -80°C prior to analysis*	☐ ☐	
		Blood put in ice box at **t=45** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	***Red serum tube (t=15) to be centrifuged.** **ORAC test to be done*
		Blood put in ice box at **t=60** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	***Red serum tube (t=30) to be centrifuged.** **ORAC test to be done*
		Centrifuge red serum tube (t=45) - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	☐ ☐	

		Blood put in ice box at **t=90** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	▢ ▢	***Red serum tube (t=60) to be centrifuged.*** **ORAC test to be done*
		At t=90 for incretins (60 & 90): - *Centrifuge EDTA tubes at 2900 rpm for 10min at 4°C* - *Aliquot samples for storage at -80°C prior to analysis*	▢ ▢	
		Blood put in ice box at **t=120** - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	▢ ▢	***Red serum tube (t=90) to be centrifuged*** **ORAC test to be done*
		At t=120 for incretin (120): - *Centrifuge EDTA tubes at 2900 rpm for 10min at 4°C* - *Aliquot samples for storage at -80°C prior to analysis*	▢ ▢	
		Centrifuge red serum tube (t=120) - Centrifuge tubes at 3500 rpm for 15min at 4°C - Aliquot samples for storage at -80°C prior to analysis	▢ ▢	

Appendix 4 – GLARE study analysis resources

The following section of the Appendices includes supplementary materials for the GLARE study data analysis and interpretation

Appendix 4.1 – Formulas of glucose and insulin indices considered in the GLARE study..274
Appendix 4.2 – Data analysis stratification options considered in the GLARE study. 280

Appendix 4.1 – Formulas of glucose and insulin indices considered in the GLARE study

Indices used in data analysis:	Indices validated by population studies to indicate risk of future T2DM	Robust measure?	Definition/ Formula	Interpretation		Reference
				Reduced risk of T2DM	**Increased risk of T2DM**	
Glucose indices						
Mean $iAUC_{glucose}$ (mmol/L.min)	Yes	Yes	Average incremental area under the curve of glucose using the trapezoidal rule. Values falling under the baseline were not included.	Lower iAUC	Higher iAUC	Wolever, T.M.S. and D.J.A. Jenkins, *The use of the glycemic index in predicting the blood glucose response to mixed meals.* American Journal of Clinical Nutrition, 1986. **43**(1): p. 167-172. Lefloch, J.P., et al., *Blood glucose area under the curve: methodological aspects.* Diabetes Care, 1990. **13**(2): p. 172-175.
1h postprandial glucose (1hPG) (mmol/L)	Yes	Yes	Glucose value at 1h OGTT	Lower 1h Glucose	Higher 1h Glucose	Buysschaert, M., et al., *An elevated 1-h post-load glucose level during the oral glucose tolerance test detects prediabetes.* Diabetes & Metabolic Syndrome-Clinical Research & Reviews, 2017. **11**(2): p. 137-139. Bergman, M., et al., *Petition to replace current OGTT criteria for diagnosing prediabetes with the 1-hour post-load plasma glucose >= 155 mg/dl (8.6 mmol/L).* Diabetes Research and Clinical Practice, 2018. **146**: p. 18-33. Pareek, M., et al., *Enhanced Predictive Capability of a 1-Hour Oral Glucose Tolerance Test: A Prospective Population-Based Cohort Study.* Diabetes Care, 2018. **41**(1): p. 171-177.
2h postprandial glucose (2hPG) (mmol/L)	Yes	Yes	Glucose value at 2h OGTT	Lower 2h Glucose	Higher 2h Glucose	Hulman, A., et al., *Heterogeneity in glucose response curves during an oral glucose tolerance test and associated cardiometabolic risk.* Endocrine,

Peak glucose value (mmol/L)	Yes	Yes	Highest glucose value during 2h OGTT	Lower peak glucose	Higher peak glucose	2017. **55**(2): p. 427-434.
Peak glucose time (min)	Yes	Yes	Time of peak glucose value	Shorter time to peak	Longer time to peak	Kramer, C.K., et al., *Emerging parameters of the insulin and glucose response on the oral glucose tolerance test: Reproducibility and implications for glucose homeostasis in individuals with and without diabetes.* Diabetes Research and Clinical Practice, 2014. **105**(1): p. 88-95.
Glucose curve shapes (Monophasic vs complex)	Yes	Yes	Glucose shapes (monophasic, biphasic or triphasic). Both biphasic and triphasic shapes are known as complex shapes	Biphasic or Triphasic (Complex)	Monophasic	Tschritter, O., et al., *Assessing the shape of the glucose curve during an oral glucose tolerance test.* Diabetes Care, 2003. **26**(4): p. 1026-1033.
Stumvoll metabolic clearance rate of glucose ($MCR_{glucose}$) (mL/kg.min)	Yes	Yes	$19.240 - 0.281 \times BMI - 0.00498 \times Insulin_{120} - 0.333 \times Glucose_{120}$ *where:* BMI (kg/m^2) $Glucose_{120}$ (mmol/L) $Insulin_{120}$ (pmol/L)	Faster rate of glucose disposal	Slower rate of glucose disposal	Stumvoll, M., et al., *Oral glucose tolerance test indexes for insulin sensitivity and secretion based on various availabilities of sampling times.* Diabetes Care, 2001. **24**(4): p. 796-797.
Insulin secretion and response indices						
***Insulin concentrations must be interpreted with insulin resistance to indicate degree of β-cell dysfunction and insulin sensitivity**						
Mean $iAUC_{insulin}$ (mU/L.min)	No	No	Average incremental area under the curve of insulin using the trapezoidal rule. Values falling under the baseline were not included.	NA	NA	Wolever, T.M.S. and D.J.A. Jenkins, *The use of the glycemic index in predicting the blood glucose response to mixed meals.* American Journal of Clinical Nutrition, 1986. **43**(1): p. 167-172. Lefloch, J.P., et al., *Blood glucose area under the curve: methodological aspects.* Diabetes Care, 1990. **13**(2): p. 172-175.
1h postprandial insulin (1hPI) (mU/L)	No	No	Insulin value at 1h OGTT	NA	NA	Crofts, C., et al., *Identifying hyperinsulinaemia in the absence of impaired glucose tolerance: An examination of the Kraft database.* Diabetes Research and Clinical Practice, 2016. **118**: p. 50-57.
2h postpeandial insulin (2hPI) (mU/L)	No	No	Insulin value at 2h OGTT	NA	NA	
Insulin peak value (mU/L)	No	No	Highest insulin value during 2h OGTT	NA	NA	
Insulin peak time	Yes	Yes	Time of peak insulin value	Shorter time to	Longer time to	Kramer, C.K., et al., *Emerging parameters of the insulin*

(min)				peak	peak	*and glucose response on the oral glucose tolerance test: Reproducibility and implications for glucose homeostasis in individuals with and without diabetes.* Diabetes Research and Clinical Practice, 2014. **105**(1): p. 88-95.
Insulin curve shapes (Monophasic vs complex) - Not validated by population studies to indicate risk of T2DM	No	No	Insulin shapes (monophasic, biphasic or triphasic) Both biphasic and triphasic shapes are known as complex shapes	NA	NA	NA
****Below are indices derived from OGTT with good correlation to hyperinsulinaemic-euglycaemic HIEC clamp studies and validated***						
			Insulin secretion			
Insulin mean incremental area under the curve / Glucose mean incremental area under the curve ($iAUC_{insulin}$ / $iAUC_{glucose}$)	Yes	Yes	$iAUC_{insulin}$ / $iAUC_{glucose}$ *where:* $iAUC_{glucose}$ (mg/dL.min) $iAUC_{insulin}$ (mU/L.min)	Higher $iAUC_{insulin}$ / $iAUC_{glucose}$	Lower $iAUC_{insulin}$ / $iAUC_{glucose}$	Levine, R. and D.E. Haft, *Carbohydrate homeostasis.* New England Journal of Medicine, 1970. **283**(5): p. 237-+.
Overall insulin sensitivity						
Matsuda index (ISI/M)	Yes	Yes	10000/(sqrt ((FBG x FI) x (Mean glucose x Mean insulin)) *where:* FBG and mean glucose (mg/dL) FI and mean insulin (mU/L) Mean glucose is calculated as (G_0 x 15+G_{30} x 30+G_{60} x 30+G_{90} x 30+G_{120} x 15)/120	Higher ISI/M	Lower ISI/M	Matsuda, M. and R.A. DeFronzo, *Insulin sensitivity indices obtained from oral glucose tolerance testing - Comparison with the euglycemic insulin clamp.* Diabetes Care, 1999. **22**(9): p. 1462-1470.

			Mean insulin is calculated as (I_0 x 15+I_{30} x 30+I_{60} x 30+I_{90} x 30+I_{120} x 15)/120			
Oral glucose insulin sensitivity (OGIS) (mL/min.m^2)	Yes	Yes	Model based on glucose and insulin physiology during an OGTT using web-based calculation: http://webmet.pd.cnr.it/ogis/ *where:* FBG, $glucose_{90}$ and $glucose_{120}$ (mg/dL) FI and $insulin_{90}$ (mU/L)	Higher OGIS	Lower OGIS	Mari, A., et al., *A model-based method for assessing insulin sensitivity from the oral glucose tolerance test.* Diabetes Care, 2001. **24**(3): p. 539-548.
Stumvoll overall insulin sensitivity ($ISI_{overall}$) (µmol/kg.min) x (pmol/L)	Yes	Yes	$0.222 - 0.00333 \times BMI - 0.0000779 \times Insulin_{120} - 0.000422 \times Age$ *where:* Age (year) BMI (kg/m^2) $Insulin_{120}$ (pmol/L)	Higher $ISI_{overall}$	Lower $ISI_{overall}$	Stumvoll, M., et al., *Oral glucose tolerance test indexes for insulin sensitivity and secretion based on various availabilities of sampling times.* Diabetes Care, 2001. **24**(4): p. 796-797.
Early phase insulin sensitivity and insulin resistance						
Quantitative insulin sensitivity check index (QUICKI) for fasting hepatic insulin sensitivity	Yes	Not as robust (Relies on fasting values and not OGTT)	1/[log(FI) + log(FBG)] *where:* FBG (mg/dL) FI (mU/L)	Higher QUICKI	Lower QUICKI	Katz, A., et al., *Quantitative insulin sensitivity check index: A simple, accurate method for assessing insulin sensitivity in humans.* Journal of Clinical Endocrinology & Metabolism, 2000. **85**(7): p. 2402-2410.
Homeostasis model assessment of (HOMA-IR) on fasting hepatic insulin resistance	Yes	Not as robust (Relies on fasting values and not OGTT)	(FBG x FI) / 22.5 *where:* FBG (mmol/L) FI (mU/L)	Lower HOMA-IR	Higher HOMA-IR	Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-419.
Stumvoll first phase of insulin response (ISI_{first}) (pmol/L)	Yes	Yes	$728 + 3.537 \times FI - 120.3 \times Glucose_{60} + 1.341 \times Insulin_{60} + 21.27 \times BMI$ *where:*	Higher ISI_{first}	Lower ISI_{first}	Stumvoll, M., et al., *Oral glucose tolerance test indexes for insulin sensitivity and secretion based on various availabilities of sampling times.* Diabetes Care, 2001. **24**(4): p. 796-797.

			BMI (kg/m^2) $Glucose_{60}$ (mmol/L) $Insulin_{60}$ (pmol/L)			
Insulinogenic index 0-30 min of OGTT for first phase of insulin response (IGI_{30})	Yes	Not as robust compared to the others but commonly used to allow for comparison with other studies	($Insulin_{30}$ - FI)/($Glucose_{30}$ - FBG) *where:* FBG and $glucose_{30}$ (mg/dL) FI and $insulin_{30}$ (mU/L)	Higher ISI_{30}	Lower ISI_{30}	Phillips, D.I.W., et al., *Understanding oral glucose tolerance. Comparison of glucose or insulin measurements during the oral glucose tolerance test with specific measurements of insulin resistance and insulin secretion.* Diabetic Medicine, 1994. **11**(3): p. 286-292.
Late phase insulin sensitivity						
Stumvoll second phase of insulin response (ISI_{second}) (pmol/L)	Yes	Yes	$208 + 0.335 \times Insulin_{60} - 26.33 \times Glucose_{60} + 0.887 \times FI + 3.933 \times BMI$ *where:* BMI (kg/m^2) $Glucose_{60}$ (mmol/L) FI and $insulin_{60}$ (pmol/L)	Higher ISI_{second}	Lower ISI_{second}	Stumvoll, M., et al., *Oral glucose tolerance test indexes for insulin sensitivity and secretion based on various availabilities of sampling times.* Diabetes Care, 2001. **24**(4): p. 796-797.
β-cell function						
Insulin-secretion-sensitivity-index (ISSI-2)	Yes	Yes	(Total $AUC_{insulin}$ / Total $AUC_{glucose}$) x ISI/M *where:* Total $AUC_{insulin}$ (mU/L.min) Total $AUC_{glucose}$ (mmol/L.min) and ISI/M uses: FBG and mean glucose (mg/dL) FI and mean insulin (mU/L)	Higher ISSI-2	Lower ISSI-2	Retnakaran, R., et al., *Hyperbolic relationship between insulin secretion and sensitivity on oral glucose tolerance test.* Obesity, 2008. **16**(8): p. 1901-1907.
Homeostasis model assessment of beta-cell function (HOMA-β)	Yes	Not as robust (Relies on fasting values and not OGTT and equation	(20 x FI)/(FBG - 3.5) *where:* FBG (mmol/L) FI (mU/L)	Higher HOMA-β	Lower HOMA-β	Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-419.

		based on healthy population)				
Oral disposition index (Insulinogenic index at 30 min of OGTT/HOMA-IR)	Yes	Yes	IGI_{30}/HOMA-IR *where:* IGI_{30} uses: FBG and $glucose_{30}$ (mg/dL) FI and $insulin_{30}$ (mU/L) and HOMA-IR uses: FBG (mmol/L) FI (mU/L)	Higher IGI_{30}/HOMA-IR	Lower IGI_{30}/HOMA-IR	Kahn, S.E., *The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes.* Diabetologia, 2003. **46**(1): p. 3-19.
Oral disposition index (Insulinogenic index at 30 min OGTT/fasting insulin)	Yes	Yes	IGI_{30}/FI *where:* IGI_{30} uses: FBG and $glucose_{30}$ (mg/dL) FI and $insulin_{30}$ (mU/L) FI (mU/L)	Higher IGI_{30}/FI	Lower IGI_{30}/FI	Utzschneider, K.M., et al., *Oral Disposition Index Predicts the Development of Future Diabetes Above and Beyond Fasting and 2-h Glucose Levels.* Diabetes Care, 2009. **32**(2): p. 335-341.

BMI, body mass index; FBG, fasting blood glucose; FI: fasting insulin; G_0: glucose value at 0 min (baseline) of the oral glucose tolerance test; G_{30}: glucose value at 30 min of the oral glucose tolerance test; G_{60}: glucose value at 60 min of the oral glucose tolerance test; G_{90}: glucose value at 90 min of the oral glucose tolerance test; G_{120}: glucose value at 120 min of the oral glucose tolerance test; $glucose_{30}$: glucose value at 30 min of the oral glucose tolerance test; $glucose_{60}$: glucose value at 60 min of the oral glucose tolerance test; $glucose_{90}$: glucose value at 90 min of the oral glucose tolerance test; $glucose_{120}$: glucose value at 120 min of the oral glucose tolerance test; I_0: insulin value at 0 min (baseline) of the oral glucose tolerance test; I_{30}: insulin value at 30 min of the oral glucose tolerance test; I_{60}: insulin value at 60 min of the oral glucose tolerance test; I_{90}: insulin value at 90 min of the oral glucose tolerance test; I_{120}: insulin value at 120 min of the oral glucose tolerance test; $iAUC_{glucose}$, glucose mean incremental area under the curve; $iAUC_{insulin}$, insulin mean incremental area under the curve; $insulin_{30}$: insulin value at 30 min of the oral glucose tolerance test; $insulin_{60}$: insulin value at 60 min of the oral glucose tolerance test; $insulin_{90}$: insulin value at 90 min of the oral glucose tolerance test; $insulin_{120}$: insulin value at 120 min of the oral glucose tolerance test; OGTT, oral glucose tolerance test; 1hPG, 1h postprandial glucose; 2hPG, 2h postprandial glucose; 1hPI, 1h postprandial insulin; 2hPI, 2h postprandial insulin.

Appendix 4.2 – Data analysis stratification options considered in the GLARE study

Stratification method	Criteria and Interpretation	Remarks	References
Single stratification			
A. Averaging	Using the mean average of each glucose and insulin index to separate participants into higher and lower groups	Arbitrary and not based on previous studies. No significant, distinct pathophysiologic patterns could be identified from this stratification. Hence this method was not chosen for stratification in the GLARE study.	NA
B. Cut off value for Matsuda index	Cut off point of <4.3 indicates reduced overall whole body insulin sensitivity (hepatic and peripheral) and indicates increased insulin resistance	n=11 participants had Matsuda index >4.3, and n=8 had an index <4.3. However, no significant, distinct pathophysiologic patterns could be identified from this stratification. Hence this method was not chosen for stratification in the GLARE study.	Gutch, M., et al., *Assessment of insulin sensitivity/resistance.* Indian Journal of Endocrinology and Metabolism, 2015. **19**(1): p. 160-164.
C. Postprandial glucose curve shapes	Monophasic shapes (only one peak) indicate higher T2DM risk compared to biphasic (2 peaks) or triphasic (2 complete peaks) shapes. Both biphasic and triphasic are also considered as complex shapes and are grouped as one.	n=11 participants exhibited monophasic glucose shapes at baseline, whilst n=8 exhibited complex glucose shapes. However, no significant, distinct pathophysiologic patterns could be identified from this stratification. Furthermore, prediabetes involves	Tschritter, O., et al., *Assessing the shape of the glucose curve during an oral glucose tolerance test.* Diabetes Care, 2003. **26**(4): p. 1026-1033.

		significant changes to insulin secretion as well so looking specifically only at glucose changes would not be sufficient. Hence this method was not chosen for stratification in the GLARE study.	
D. Combined postprandial glucose and insulin peak time patterns (Takahashi study)	Increased glucose and insulin peak times indicate higher degree of impaired glucose metabolism and increased risk of T2DM. Research has elucidated that early insulin defects (causing delayed insulin peak) might lead to hyperinsulinaemia, and also worsened glycaemic control. There are four patterns in total: Pattern 1: Normal glucose and insulin peaking at 30min Pattern 2: Normal glucose peaking at 30 min but insulin late >30min Pattern 3: Both glucose and insulin late >30min Pattern 4: Insulin very late >60min Participants exhibiting patterns 1and 2 at baseline were classified as the	n=9 participants exhibited less healthy patterns 3 and 4 at baseline, whilst n=11 exhibited healthier patterns 1 and 2. There were significant, distinct pathophysiologic patterns identified in the two groups classified by their postprandial glucose and insulin peak time patterns. This stratification method was also superior to just using the glucose shapes to classify participants. Hence this stratification method was chosen to be used for the GLARE study.	Refer to chapter 5 on the GLARE study Takahashi, K., et al., *Four Plasma Glucose and Insulin Responses to a 75g OGTT in Healthy Young Japanese Women.* Journal of Diabetes Research, 2018: p. 7.

	healthier group, whilst participants exhibiting patterns 3 and 4 at baseline were classified as the less healthy group.		
E. Normal and hyperinsulinaemic groups (Hayashi and Kraft insulin patterns)	According to Hayashi and Kraft studies, individuals at higher risk of T2DM have higher than normal secretion of insulin. Pattern 1 is considered to be having less abnormality in insulin response than subsequent patterns. Pattern 5 exhibits the worst insulin response abnormality. Kraft patterns: Generally five patterns (However only four patterns were applicable to the GLARE study as pattern 5 is for diabetic, hypoinsulinaemic individuals): Pattern 1: Normal insulin (Fasting insulin ≤30 mU/L AND 30 min or 1h peak) Pattern 2: Borderline hyperinsulinaemic (Fasting insulin ≤50 mU/L AND 30 min or 1h peak) Pattern 3: Hyperinsulinaemia (Fasting insulin ≤50 mU/L AND delayed peak (2h)) Pattern 4: Hyperinsulinaemia (Fasting insulin >50 mU/L)	Using Kraft patterns, GLARE participants only exhibited patterns 1-3. However, all participants had fasting insulin concentrations <30 mU/L, which made accurate classification into the respective categories difficult. Hence this method was not chosen for stratification in the GLARE study.	Hayashi, T., et al., *Patterns of Insulin Concentration During the OGTT Predict the Risk of Type 2 Diabetes in Japanese Americans.* Diabetes Care, 2013. **36**(5): p. 1229-1235. Crofts, C., et al., *Identifying hyperinsulinaemia in the absence of impaired glucose tolerance: An examination of the Kraft database.* Diabetes Research and Clinical Practice, 2016. **118**: p. 50-57.

<table>
<tr><td></td><td>Hayashi patterns:
Pattern 1: Peak insulin at 30 min, with 60 min >120 min

Pattern 2: Peak insulin peak at 30 min, with 60 min ≤120 min

Pattern 3: Peak insulin at 60 min

Pattern 4: Peak insulin at 120 min, with 30min <60 min

Pattern 5: Peak insulin at 120 min, with 30 min ≥60 min</td><td>Using Hayashi patterns, GLARE participants (n=9) exhibited peak insulin at 90 min or 120 min, with participants (n=10) having peak insulin at 30 min or 60 min.

However, as Hayashi patterns do not consider insulin peaks at 90 min, this method was eventually not used. However it may be interesting to observe for the impact of the tested plant extracts on participants (n=9) with delayed insulin responses (≥ 90 min).</td><td></td></tr>
<tr><td colspan="4">Combined stratification (using two methods of stratification)</td></tr>
<tr><td>C + D</td><td>Categorising participants with double risk factors for T2DM (Monophasic and delayed glucose and insulin peak times)</td><td rowspan="3">It was decided that combining two stratification methods might make data analysis too complex and so single stratification was preferred. Moreover no previous research has conducted a combination of two stratification methods although it was useful to do a preliminary exploration of each of these stratification methods.

Hence these methods were not chosen for stratification in the GLARE study.</td><td>NA</td></tr>
<tr><td>C + E</td><td>Categorising participants with double risk factors for T2DM (Monophasic and hyperinsulinaemic)</td><td>NA</td></tr>
<tr><td>D + E</td><td>Categorising participants with double risk factors for T2DM (Hyperinsulinaemic and delayed glucose and insulin peak times)</td><td>NA</td></tr>
</table>

T2DM: Type 2 diabetes mellitus.

Appendix 5 – DRC 16 forms

The following section of the Appendices includes the DRC 16 forms that have been acknowledged and signed by the primary supervisor, Assoc Prof Rachel A. Page

Appendix 5.1 – DRC 16 form for Chapter 3 of the thesis 285
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Appendix 5.1 – DRC 16 form for Chapter 3 of the thesis

DRC 16

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Wen Xin Janice Lim
Name/title of Primary Supervisor:	A/P Rachel Page
In which chapter is the manuscript /published work:	Chapter 3

Please select one of the following three options:

◯ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

◯ The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	[signature]
Date:	09/10/20
Primary Supervisor's Signature:	[signature]
Date:	12/10/20

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Appendix 5.2 – DRC 16 form for Chapter 4 of the thesis

DRC 16

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In which chapter is the manuscript /published work:	Chapter 4

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- Please provide the full reference of the Research Output:

Lim, W.X.J., et al., An Acute, Placebo-Controlled, Single-Blind, Crossover, Dose-Response, Exploratory Study to Assess the Effects of New Zealand Pine Bark Extract (Enzogenol (R)) on Glycaemic Responses in Healthy Participants. Nutrients, 2020. 12(2): p. 14.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

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Appendix 5.3 – DRC 16 form for Chapter 5 of the thesis

DRC 16

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Appendix 5.4 – DRC 16 form for Chapter 6 of the thesis

DRC 16

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