

**Antidiabetic properties of anthocyanins - *in vitro* and *in vivo*  
studies**

Sadia Zulfiqar

Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Leeds

School of Food Science and Nutrition

February 2022

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

## **List of Conference abstracts accepted**

### **Poster presentation**

S. Zulfiqar, L. Marshall, and C. Bösch. Comparison of *in vitro* assays to determine inhibition of  $\alpha$ -amylase enzyme activity of anthocyanins. XXX International Conference on Polyphenols (ICP 2020), Turku, Finland.

S. Zulfiqar, L. Marshall, and C. Bösch. *In vitro* inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase by *Hibiscus sabdariffa*. The 10<sup>th</sup> International Workshop on Anthocyanins (IWA 2019), San Michele all'Adige, Italy.

S. Zulfiqar, K. Benton, T. Hassan, L. Marshall, and C. Bösch. *In vitro* and *in vivo* anti-diabetic properties of *Hibiscus sabdariffa*. "Inter-individual differences in the nutrition response" The Nutrition Society Spring Conference (2019), Dundee, UK.

### **Publications**

S. Zulfiqar, L. Marshall, and C. Bösch. *Hibiscus sabdariffa* inhibits  $\alpha$ -glucosidase activity *in vitro* and lowers postprandial blood glucose response in humans (Submitted to Journal of Human Nutrition & Metabolism-October 2021).

S. Zulfiqar, F. Blando, M. Benohoud, C. Orfila, L. Marshall, and C. Bösch. Comparison of the direct chromogenic assay with 3,5-dinitrosalicylic acid (DNS) assay to determine  $\alpha$ -amylase inhibitory properties in pigmented samples (Currently being updated for submission to Journal of Food Technology-February 2022).

S. Zulfiqar, L. Marshall, and C. Bösch. *In vitro* and *in vivo* antidiabetic properties of blueberry concentrate - a dose dependent response (in preparation).

*This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement*

@2022 The University of Leeds and Sadia Zulfiqar

## **Acknowledgements**

First of all, I would like to thank God, the Almighty, for His countless blessings and courage throughout my research work. Undertaking PhD has been a truly life-changing experience for me, and it would not have been possible to complete my research work without the support and guidance that I received from many people. I will be extremely grateful to my principal supervisor Dr. Christine Bösch for all her guidance, consistent support, encouragement, tolerance and as well as motivation throughout my PhD journey. Thank you for boosting my confidence and enabling me to pursue my dreams. I sincerely appreciate Dr. Lisa Marshall for giving me positive feedback and for her kind-hearted supervision and support throughout the whole period of this research.

My sincere thanks also goes to all the amazing staff and colleagues who helped me directly or indirectly during this period, Miles Ratcliffe, Sara Viney, Ian Hardy and Neil Rigby for helping and providing me technical support. I would like to thank my PhD colleagues who have always supported and encouraged me: Catherine, Shirley, Maryam, Ruixian, Kartika, Suvro, and Ng'andwe. I am sincerely grateful and thankful to my best friend, Sumali for her kindness, support, and attentiveness to my problems and helping me in finding solutions. I cannot forget to pay gratitude to my family, especially, my father and sisters for believing in me and providing the unconditional love and emotional support throughout my PhD. Special thanks to my brother Junaid and brother-in-law Tahir Ali who supported me financially and emotionally.

And finally, I would like to express my endless gratitude to my husband, Ahmad and my PhD baby Rameen, for their love, understanding, prayers and continuing emotional support to complete this research work. Thank you so much Ahmad for believing in me and encouraging me during these 4 years. I won't be in this position without your support.

## Abstract

Reducing postprandial hyperglycaemia by attenuating carbohydrate digestion is an effective tool in the prevention and management of T2DM. In the past few decades, a huge deal of scientific effort was put into identifying natural plant derived ingredients as anti-diabetic agents with fewer side effects. Anthocyanins have been highlighted for their potential to lower blood glucose levels by inhibition of carbohydrate digesting enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase, however, it has been recognized that the diversity of anthocyanins from different sources may differently contribute to anti-diabetic effects.

This research investigated the acute effects of anthocyanin-rich *Hibiscus sabdariffa* (HS) intake on glycaemic response *in vivo* and *in vitro*. The outcomes were compared with blueberry, a more common source of anthocyanins which has demonstrated evidence for its hypoglycaemic effects. Therefore, two intervention studies were conducted on healthy subjects to determine the dose-response effects of anthocyanins on glycaemic response using either blueberry or hibiscus. *In vitro* inhibition assays were performed to compare the inhibitory effects of anthocyanin-rich extracts and pure compounds, against and with acarbose as a synthetic inhibitor, on  $\alpha$ -amylase and  $\alpha$ -glucosidase enzyme activities.

HS dose-dependently inhibited  $\alpha$ -glucosidase activity *in vitro* and reduced postprandial blood glucose levels in healthy volunteers, indicating its hypoglycaemic effects. Blueberry had stronger *in vivo* postprandial effects and *in vitro* inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase compared to HS. The *in vivo* data indicate that anthocyanin-rich drinks/foods have a reduced glycaemic response that differs with anthocyanin dose and source

In summary, the present data indicate that consumption of anthocyanin-rich foods exerts beneficial effects on postprandial glucose response that may be attributed to their enzyme inhibitory mechanisms. The present study identified that inhibitory properties of anthocyanins influenced by a number of factors including different anthocyanidin structure/glycosylation, differential inhibition of amylase and glucosidase and presence of other components.

## Table of content

<b>Acknowledgements</b> .....	<b>ii</b>
<b>Abstract</b> .....	<b>iii</b>
<b>List of Tables</b> .....	<b>viii</b>
<b>List of Figures</b> .....	<b>ix</b>
<b>List of abbreviations</b> .....	<b>xii</b>
<b>Chapter 1 Introduction and literature review</b> .....	<b>1</b>
1.1 Introduction .....	1
1.2 Digestion and absorption of carbohydrates.....	2
1.3 Glycaemic index .....	3
1.4 Diabetes and glucose homeostasis .....	4
1.5 Polyphenols.....	5
1.6 Anthocyanin sources and intake.....	5
1.7 Beneficial effects of anthocyanins towards diabetes.....	7
1.7.1 Role of anthocyanins in IR.....	8
1.7.2 Role of anthocyanins in pancreatic $\beta$ -cell protection .....	11
1.7.3 Inhibition of carbohydrate digesting enzymes .....	11
1.8 Mode of carbohydrate digesting enzyme inhibition .....	14
1.9 Anthocyanins and postprandial glycaemia.....	15
1.10 <i>Hibiscus sabdariffa</i> .....	16
1.11 Aims and objectives.....	25
<b>Chapter 2 Materials and methods</b> .....	<b>26</b>
2.1 Materials.....	26
2.1.1 Chemicals and reagents.....	26
2.1.2 Sample preparation .....	27
2.2 Analytical methods .....	27
2.2.1 Total phenolic content assay .....	27
2.2.2 Total monomeric anthocyanins by pH differential method.....	28
2.2.3 Determination of anthocyanins by HPLC\LC-MS .....	29
2.2.4 Determination of sugars using HPLC-evaporative light scattering detector (ELSD) .....	30
2.3 Biochemical assays.....	31
2.3.1 Determination of $\alpha$ -glucosidase inhibition .....	31
2.3.2 Determination of $\alpha$ -amylase inhibition.....	34

2.3.3	Enzymatic kinetics and mode of inhibition .....	36
2.4	<i>In vivo</i> human glycaemic study (Hibiscus and blueberry study) .....	37
2.4.1	Materials.....	37
2.4.2	Ethical approval.....	37
2.4.3	Participant recruitment.....	37
2.4.4	Preparation of test drinks.....	38
2.4.5	Blood sampling .....	38
2.4.6	Study protocol .....	39
2.4.7	Insulin measurement by ELISA .....	40
2.5	Statistical analysis .....	40
<b>Chapter 3 <i>Hibiscus sabdariffa</i> inhibits <math>\alpha</math>-glucosidase activity <i>in vitro</i> and lowers postprandial blood glucose response in humans .....</b>		<b>42</b>
3.1	Abstract .....	42
3.2	Introduction .....	43
3.3	Aims and objectives.....	44
3.4	Objectives .....	45
3.5	Materials and Methods .....	45
3.5.1	Compositional analysis.....	45
3.6	Study design.....	45
3.6.1	Control and intervention drinks .....	45
3.7	Human study: subjects and design.....	46
3.8	Statistical analysis .....	47
3.9	<i>In vitro</i> enzyme inhibition .....	48
3.10	Results .....	48
3.10.1	Compositional analysis of hibiscus sample.....	48
3.10.2	Human study: glycaemic response .....	50
3.10.3	Inhibition of $\alpha$ -glucosidase and $\alpha$ -amylase <i>in vitro</i> .....	53
3.11	Discussion.....	57
3.12	Conclusion .....	62
<b>Chapter 4 Comparison of a direct chromogenic assay with 3,5-dinitrosalicylic acid (DNS) assay to determine <math>\alpha</math>-amylase inhibitory properties in pigmented samples .....</b>		<b>63</b>
4.1	Abstract .....	63
4.2	Introduction .....	63
4.3	Aims .....	66
4.4	Objectives .....	66

4.5	Materials and methods .....	67
4.6	Results .....	68
4.6.1	Total polyphenol, total anthocyanin and total sugar contents of fruit extracts.....	68
4.6.2	Comparison of DNS and direct chromogenic assay for $\alpha$ -amylase inhibition by acarbose.....	69
4.6.3	Enzyme inhibitory effects of anthocyanin containing extracts in DNS and chromogenic assay .....	71
4.6.4	Application of chromogenic assay to determine $\alpha$ -amylase inhibition in crude and pure extracts .....	74
4.6.5	Mode of inhibition of anthocyanins on $\alpha$ -amylase activity.....	74
4.6.6	$\alpha$ -glucosidase inhibition by anthocyanin containing extracts and pure compounds.....	76
4.7	Discussion.....	76
4.8	Conclusion .....	81
<b>Chapter 5 <i>In vitro</i> and <i>in vivo</i> antidiabetic properties of blueberry concentrate- a dose dependent effect/response .....</b>		<b>83</b>
5.1	Abstract.....	83
5.2	Introduction .....	84
5.3	Aims and objectives.....	86
5.4	Materials and methods .....	86
5.4.1	Reagents and samples.....	86
5.4.2	Characterization of blueberry samples (Total phenolic, monomeric anthocyanins and sugar content) .....	86
5.4.3	<i>In vivo</i> human study.....	87
5.4.4	<i>In vitro</i> enzyme inhibition .....	89
5.5	Results .....	90
5.5.1	Compositional data of blueberry juice .....	90
5.5.2	Effect of blueberry on postprandial plasma glucose response .....	90
5.5.3	<i>In vitro</i> enzyme inhibition .....	92
5.6	Discussion.....	97
5.7	Limitations of study.....	100
5.8	Conclusion .....	100
<b>Chapter 6 General discussion.....</b>		<b>102</b>
6.1	<i>In vivo</i> postprandial effects of <i>Hibiscus sabdariffa</i> .....	103
6.2	<i>In vitro</i> carbohydrate inhibition.....	106
6.3	Impact and implication of this research.....	108

6.4 Conclusion and future perspectives.....	110
<b>References .....</b>	<b>112</b>
<b>List of appendixes .....</b>	<b>128</b>
<b>Appendix A.....</b>	<b>128</b>
<b>Appendix B.....</b>	<b>130</b>
<b>Appendix C.....</b>	<b>133</b>
<b>Appendix D.....</b>	<b>136</b>
<b>Appendix E .....</b>	<b>137</b>

## List of Tables

Table 1.1 Summary of cell culture studies showing different effects of <i>Hibiscus sabdariffa</i> .....	19
Table 1.2 Summary of animal studies showing different health effects of <i>Hibiscus sabdariffa</i> .....	20
Table 1.3 Summary of human intervention studies showing different health effects of <i>Hibiscus sabdariffa</i> .....	22
Table 1.4 <i>In vitro</i> $\alpha$ -glucosidase inhibition by <i>Hibiscus sabdariffa</i> .....	23
Table 1.5 <i>In vitro</i> $\alpha$ -amylase inhibition by <i>Hibiscus sabdariffa</i> .....	24
Table 3.1 Characteristics/composition of test meals/drinks .....	46
Table 3.2 Compositional analysis of HS concentrate .....	49
Table 3.3 Characteristics of participants .....	50
Table 4.1 Total polyphenols and total anthocyanins in fruit extracts .....	68
Table 4.2 Sugar analysis of fruit extracts by HPLC-ELSD .....	68
Table 4.3 Comparison of $\alpha$ -amylase inhibitory properties in anthocyanin-rich extracts and individual anthocyanins using direct chromogenic assay versus DNS assay .....	73
Table 4.4 Summary of studies measuring $\alpha$ -amylase inhibition by pure anthocyanins and anthocyanin-rich samples via DNS assay .....	78
Table 4.5 Summary of studies measuring $\alpha$ -amylase inhibition by pure anthocyanins and anthocyanin-rich samples via direct chromogenic assay .....	79
Table 5.1 Characteristics of the subjects at baseline .....	87
Table 5.2 Characteristics of tested meals/drinks .....	88
Table 5.3 The IC <sub>50</sub> ( $\mu$ g/mL) values of blueberry against $\alpha$ -glucosidase, and pancreatic $\alpha$ -amylase .....	96

## List of Figures

Figure 1.1 Basic structure of anthocyanins .....	6
Figure 1.2 The different mechanisms responsible for protective role of anthocyanins in preventing and managing T2DM (Les et al., 2021). .....	8
Figure 2.1 $\alpha$ -glucosidase measurement in HS samples with colour control. ....	32
Figure 2.2 Wavelength scans of product formation and colour control samples of different dilutions (A-F high to lower) of HS under same experimental conditions. ....	33
Figure 3.1 Human study design .....	47
Figure 3.2 $\alpha$ -amylase inhibition in HS samples.....	48
Figure 3.3 Typical HPLC/MS chromatogram ( $\lambda = 520$ nm) of anthocyanins distribution in <i>Hibiscus sabdariffa</i> . Peaks refer to the following compounds: 1: delphinidin sambubioside, 2: delphinidin glucoside, 3: cyanidin glucoside, 4: cyanidin sambubioside.....	49
Figure 3.4 Incremental blood glucose response over 180 min following consumption of control drink (●) and either low (■) or high dose hibiscus (▲) sugar matched drinks (A); as well as peak plasma glucose (B) and total iAUC (C). The data represent means with SEM of 15 participants. Post hoc analysis of time-point differences in change from baseline in glucose with Tukey's adjustment at $P < 0.05$ was done for control compared with low and high dose of hibiscus. The symbols * and # indicate the significant difference between control and low and high dose of hibiscus, respectively.....	51
Figure 3.5 Incremental plasma insulin response over 180 min following consumption of control drink (●) and either low (■) or high dose hibiscus (▲) sugar matched drinks (A). (B) indicates plasma peak insulin concentrations and (C) total iAUC. Data are mean with SEM of 9 participants. Post hoc analysis of time-point differences for change of insulin, with Tukey's adjustment at $P < 0.05$ was done for control compared with low and high dose of hibiscus. The symbols * indicates the significant difference between control and high dose of hibiscus. No significant difference ( $P > 0.05$ ) was observed after intake of low dose of hibiscus as compared to control.....	52
Figure 3.6 Dose dependent inhibition of $\alpha$ -glucosidase enzyme by acarbose (A) ranging from 0-4000 $\mu\text{g}/\text{mL}$ and HS extract (B) ranging from 0-400 $\mu\text{g}$ polyphenols /mL. The results are expressed as mean with SEM of three independent measurements performed in duplicate. Kinetic measurement of $\alpha$ -glucosidase activity inhibition for acarbose (C, D) and HS extract (E, F) are presented. Data recording was performed per minute over a total period of 10 min (B, E) followed by wavelength scan (C, F) of each sample in the visible range (350-600 nm) to confirm reaction product p-nitrophenol. Shown is a representative set of data within one experiment. ....	55

Figure 3.7 Effect of different concentrations of hibiscus polyphenols and acarbose on $\alpha$ -glucosidase inhibition (A). The % inhibition at selected concentrations; 191.5 $\mu$ g polyphenols/mL HS extract + 1625 $\mu$ g/mL acarbose (B) and 95.7 $\mu$ g polyphenols/mL HS extract + 812.5 $\mu$ g/mL acarbose (C) is increased. Data are mean with SEM of three experiments performed in duplicate. * indicates significant difference of hibiscus-acarbose combination versus acarbose ( $P < 0.05$ , t-test).....	56
Figure 4.1 DNS assay for measuring $\alpha$ -amylase inhibition .....	67
Figure 4.2 Direct chromogenic assay for measuring $\alpha$ -amylase inhibition ....	67
Figure 4.3 Reaction progress curves at different concentration of substrate (using 1 U/mL of PPA) indication linearity of the reaction (A) and the Lineweaver-Burk double reciprocal plot (B). .....	70
Figure 4.4 Dose-dependent effect of acarbose on inhibition of $\alpha$ -amylase determined by (A) DNS assay using amylose as substrate and (B) by direct chromogenic assay using CNPG3 as a substrate. The kinetic data of the measurement (C) and wavelength scan (D) demonstrate the linear relationship of absorbance with substrate cleavage/product formation at 405 nm over time, and nitrophenol product formation at different concentrations of acarbose, respectively. ....	70
Figure 4.5 Dose-dependent $\alpha$ -amylase inhibition by blackcurrant determined by (A) DNS assay (0-1000 $\mu$ g/mL) and (B) direct chromogenic assay (0-500 $\mu$ g/mL). Substrate cleavage and product formation were monitored through kinetic measurement recording (B) and wavelength scan (C). ....	72
Figure 4.6 Lineweaver–Burk plots demonstrating inhibition type of (A) acarbose, (B) cyanidin-3-O-galactoglucoside and (C) blackcurrant against pancreatic $\alpha$ -amylase.....	75
Figure 5.1 Postprandial blood glucose responses of 9 healthy participants after consumption of blueberry juice. Mean ( $\pm$ SEM) incremental changes in glucose concentrations (A) in response to equal amounts of carbohydrate from white wheat bread consumed with either sugar matched control (C), low dose (D1) or high dose (D2) of blueberry concentrate. Time points at which statistically significant differences were observed (two-way ANOVA; multiple comparison Tukey’s test) are identified by * symbol. Individual changes in iAUC (B) were also presented. The total iAUC was reduced significantly ( $P < 0.05$ ) by both doses of blueberry drink (C). ....	91
Figure 5.2 The dose dependent inhibitory effects of BJ polyphenols on $\alpha$ -glucosidase (A) and pancreatic $\alpha$ -amylase (B). Lineweaver–Burk plots of $\alpha$ -glucosidase (C) and $\alpha$ -amylase (D). The results are expressed as means with SEM of three independent measurements performed in duplicate.....	94

**Figure 5.3 The kinetic measurement and wavelength scan (350-600 nm) of  $\alpha$ -glucosidase (A, C) and  $\alpha$ -amylase (B, D) activity inhibition for blueberry. Shown is a representative set of data within one experiment..... 96**

## List of abbreviations

ALX	Alloxan
AMPK	AMP-activated protein kinase
ANS	3-amino-5-nitrosalicylic acid
AUC	Area under the curve
BJ	Blueberry juice
BMI	Body mass index
CNPG3	2-chloro-4 nitrophenyl $\alpha$ -D-maltotrioxide
CS	Cyanidin-3-O-sambubioside
CVD	Cardiovascular disease
DCCT	Diabetes Control and Complication Trail
DM	Diabetes mellitus
DPP-IV	Dipeptidyl peptidase IV.
DS	Delphinidin-3-O-sambubioside
ELSD	Evaporative light scattering detector
FBG	Fasting blood glucose
GA	Gallic acid
GI	Glycaemic index
GLUT2	Glucose transporter 2
GLUT4	Insulin-responsive transporter
HFD	High fat diet
HPAE-PAD	High-Performance Anion-Exchange chromatography coupled with Pulsed Amperometric Detector
HPLC	High performance liquid chromatography
HPLC/MS	High performance liquid chromatography/mass spectrophotometry
HS	Hibiscus sabdariffa
IC <sub>50</sub>	Half maximal inhibitory concentration
IL-6	Interleukin 6

IPAQ	International Physical Activity Questionnaire
IR	Insulin resistance
ITT	Insulin tolerance test
KCl	Potassium chloride
LC	Liquid chromatography
MCP-1	Monocyte chemoattractant protein-1
NCDs	Non-communicable diseases
-OH	Hydroxyl group
PBS	Phosphate buffer saline
<i>p</i> NPG	<i>p</i> -nitrophenyl glucopyranoside
PP	Polyphenol
PPA	Porcine pancreatic amylase
PPAR receptor- $\gamma$	Proliferator-activated receptor- $\gamma$
ROS	Reactive oxygen species
SEM	Standard error of the mean
SGLT1	Sodium-dependent transporter
SOD	Superoxide dismutase
SPE	Solid phase extraction
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
UKPDS	United Kingdom Prospective Diabetes Study
UV/VIS	Ultraviolet/visible range

# Chapter 1

## Introduction and literature review

### 1.1 Introduction

Diabetes mellitus (DM) is a chronic, metabolic disorder which is a leading cause of mortality worldwide. Currently, a total of 463 million adults have been reported as diabetic, and the incidence is projected to increase to 700 million in 2045 (Saeedi et al., 2019). Together with other non-communicable diseases (NCDs) such as cardiovascular disease, cancer, and respiratory disease, diabetes accounts for over 80% of premature deaths (Patterson et al., 2019). Diabetes complications can lead to blindness, kidney failure, heart attack, stroke, and lower limb amputation (Tripathi & Srivastava, 2006).

The majority of diabetic patients can be classified into one of the two broad categories: type 1 and Type 2 diabetes. Type 1 or juvenile-onset diabetes, also known as insulin dependent diabetes, is an autoimmune genetic disorder, caused by insulin deficiency due to pancreatic  $\beta$ -cells destruction. The patients with Type 1 diabetes require external insulin (tablet/injection) to maintain their blood glucose levels. Type 2 diabetes mellitus (T2DM) is typically adult-onset diabetes or insulin independent form of diabetes, characterised by elevated blood glucose levels known as hyperglycaemia. The reason for T2DM is insufficient secretion of insulin from the  $\beta$ -cells of the pancreatic islets, coupled with impaired insulin action in target tissues such as muscle, liver and fat (a condition termed as insulin resistance). T2DM is the most common form of diabetes mellitus, accounting for 90% to 95% of all diabetic patients (Tripathi & Srivastava, 2006) and its prevalence is raising worldwide. There are several classes of drugs available for treating hyperglycaemia, including sulfonylureas such as glipizide which enhances insulin secretion from the pancreas, biguanides such as metformin (decrease hepatic gluconeogenesis); peroxisome proliferator-activated receptor- $\gamma$  (PPAR receptor- $\gamma$ ) agonists (sensitize insulin); and  $\alpha$ -glucosidase inhibitors such as acarbose (reduce the glucose absorption in the intestine). These drugs are

administered either individually or in combination with other hypoglycaemic agents (Chaudhury et al., 2017; Rehani et al., 2019).

Given that T2DM is more moderate than Type 1 and can be managed by controlling the blood glucose levels, many people with T2DM may not necessarily need medication or insulin therapy and could control T2DM by making lifestyle changes such as eating a healthy diet, exercising regularly, and losing weight. Metformin is the first-line medication for people with T2DM who have difficulty in maintaining their blood glucose level (Diabetes Prevention Program, 2012). Many patients with T2DM may require insulin at some point when oral hypoglycaemic agents might not be effective any more (American Diabetes, 2010). Given the side effects associated with these drugs and insufficient glycaemic control in many diabetic patients, changes in lifestyle, such as increase in physical exercise and balanced diet both with lowest adverse side effects, are presumed to be the most promising approaches to prevent or delay the onset of T2DM.

## **1.2 Digestion and absorption of carbohydrates**

Carbohydrates are one of the macronutrients in the human diet and important for the provision of energy to support body functions (Jéquier, 1994). Dietary carbohydrates are classified as mono, di, oligo and polysaccharides. The most important sources of carbohydrates in the human diet are starch and sucrose. These carbohydrates are hydrolysed by the combined actions of amylases (salivary and pancreatic) and glucosidases (intestinal sucrase and maltase), with glucose as the main monosaccharide (Hanhineva et al., 2010). The main transporters for glucose absorption in the small intestine are sodium/glucose cotransporter 1 (SGLT1) and Glucose transporter 2 (GLUT2). In healthy conditions, glucose is transported across the apical membrane of enterocytes via active transport through SGLT1, and its exit into the portal circulation is enabled by facilitated diffusion through GLUT2, located at the basolateral membrane (Chen et al., 2016). At high luminal glucose concentrations, glucose absorption in the small intestine can be influenced by other mechanisms. It has been reported that at high carbohydrate loads, the GLUT2

transporters can be quickly incorporated into the brush border membrane of enterocytes and participate in facilitated diffusion of glucose across this membrane (Williamson, 2013; Gromova et al., 2021)

The postprandial increase in blood glucose levels is determined by the difference between the amount of the glucose entering and leaving the circulation. Under healthy conditions, glucose levels are maintained at homeostatic levels (4–6 mmol L<sup>-1</sup>) by pancreatic hormonal control (glucagon, insulin, adrenaline). In response to elevated glucose levels in the blood,  $\beta$ -cells of the pancreas secrete the hormone insulin, which stimulates the uptake of glucose through increased presence of GLUT4 glucose uptake transporter, into peripheral tissues such as muscle and adipose tissue. The anabolic action of insulin also promotes the storage of glucose in the liver as glycogen and prevents lipolysis in adipose tissue. In contrast, glucagon is secreted by pancreatic  $\alpha$ -cells in response to low blood glucose levels which has catabolic action and induces glycogenolysis as well as gluconeogenesis ensuring sufficient glucose in circulation to provide energy for the body functions (Röder et al., 2016). Both these hormones work antagonistically and regulate blood glucose concentrations. In healthy individuals, the fasting blood glucose levels range from 3.9–5.4 mmol/L and postprandial glucose levels rises up to 7.7 mmol/L which return to baseline after 2–3-hour post meal consumption (Leahy et al., 2019; Dimitriadis et al., 2021). The carbohydrate digestion occurs quite rapidly in the gastrointestinal tract in the absence of hindrances from other food components. However, the post meal increase in blood glucose depends on the source and physical state of the starch (Venn et al., 2014). The glycaemic rising capacity of dietary carbohydrates can be determined by measuring their glycaemic index (GI).

### **1.3 Glycaemic index**

Glycaemic index is a measure of the glycaemic response caused by fixed amounts of available carbohydrates from a test food to the same amount of available carbohydrates from a standard food (glucose or white bread) consumed by the same subject (Jenkins et al., 2002). The GI determines the increase in the area under the blood glucose response curve

of a 50 g carbohydrate portion of a test food over 2 hours and is expressed as the percentage of the response of the test food, to the response of a standard food of the same amount consumed by the same person. The glycaemic index of glucose is set at 100 and other foods are presented relative to this.

Generally, there are three categories of foods based on their GI values: The high-GI foods (> 70), intermediate-GI foods (>55 – < 70) and low-GI foods (< 55). Foods that are digested and absorbed slowly, are low GI foods and those that are digested and absorbed moderately are intermediate GI foods. The high GI foods are digested and absorbed rapidly and elevate blood glucose levels most (Eleazu, 2016). Investigations have shown that intake of low GI foods or having components in the diet that can lower the GI of foods, can lower the risk of certain chronic diseases such as cardiovascular disease and diabetes. Similarly, regular consumption of high glycaemic index foods plays a role in the development of chronic diseases like T2DM, obesity and cardiovascular disease (Jenkins et al., 2002).

#### **1.4 Diabetes and glucose homeostasis**

Diet plays a crucial role in the management of diabetes. Carbohydrates present in the diet are the main source of energy for the humans. Digestion of carbohydrates leads to increased postprandial blood glucose levels. The consumption of high GI foods, such as potatoes and white bread, which contain highly bioavailable starch, can cause rapid increase in blood sugar and insulin levels, thus contributing to insulin resistance. Persistent elevated blood glucose level (>7.7 mmol/L) leads to hyperglycaemia, a risk factor associated with developing T2DM (Hanhineva et al., 2010). Chronically high circulating glucose levels in the blood can lead to insulin resistance (IR), which is defined as impaired ability of cells (muscle, liver and fat) to absorb and use blood glucose for energy. Hyperglycaemia stimulates the pancreatic cells to release more insulin to compensate the excessive blood glucose. The chronic glucose stimulus for insulin secretion eventually leads to  $\beta$ -cell failure which characterises the T2DM (Kahn et al., 2006). Therefore, strict glycaemic control is the hallmark in the prevention and management of T2DM (Williamson, 2013).

Regulation of blood glucose level by controlling postprandial hyperglycaemia is a key mechanism to control or manage T2DM. Carbohydrate digestion starts with salivary  $\alpha$ -amylase in the mouth, hydrolysing  $\alpha$ -1,4-bonds in starch into maltose and dextrin then being converted to glucose by intestinal brush border  $\alpha$ -glucosidase enzymes and absorbed in the human intestinal cells (Williamson, 2013). Inhibition of these enzymes leads to reduced carbohydrate degradation and glucose intestinal absorption; thereby, reducing postprandial hyperglycaemia after consuming a meal.

## **1.5 Polyphenols**

Polyphenols are a large and heterogeneous group of plant secondary metabolites present in fruits (such as berries, grapes, cherries, apples), vegetables (particularly, broccoli, onion and cabbage), legumes, tea and coffee. Based on the number of phenol rings and structures, polyphenols are mainly classified into phenolic acids, flavonoids, stilbenes and lignans (Scalbert et al., 2005). Intake of diets rich in fruits and vegetables have been linked with reduced risk of major chronic diseases including diabetes, cardiovascular diseases and cancer (Vita, 2005; Kim et al., 2016; Briguglio et al., 2020). Anthocyanins belong to the flavonoid subgroup of polyphenols, gaining significant attention of scientific research due to recent evidence of their beneficial effects on health. Increasing evidence from epidemiological studies has demonstrated an inverse association between consumption of anthocyanin-rich foods and the risk of diabetes (Turrini et al., 2017; Putta et al., 2018). Possible mechanisms by which these polyphenolic compounds impact diabetes have been investigated through *in vitro* and *in vivo* studies (Les et al., 2021). The following section is focused on anthocyanin sources and their consumption and effects of dietary anthocyanins on diabetes.

## **1.6 Anthocyanin sources and intake**

Anthocyanins are water-soluble natural pigments present in many fruits and vegetables (Wallace & Giusti, 2015). They differ with respect to their anthocyanidin (aglycone of

anthocyanins) skeleton, type of sugars and potential aliphatic and aromatic acyl moieties, and their substitution positions. The basic structure of anthocyanins is shown in Figure 1.1.; it consists of an anthocyanidin (aglycone) with one or more sugar moieties linked to the hydroxyl groups at the 3 and/or 5 position on the C-ring (Bueno et al., 2012). Anthocyanins are accountable for blue, purple or red colors in fruits, vegetables, flowers, leaves, and grains. The six more prevalent anthocyanins found in fruits and vegetables are cyanidin (50%), delphinidin (12%), malvidin (12%), pelargonidin (12%), peonidin (7%) and petunidin (7%) (Khoo et al., 2017).

As part of a typical diet, humans consume considerable amounts of anthocyanins through fruits and fruit-based products (e.g. berries, jam, juice, wine), vegetables and cereals (e.g. eggplant, red onion, red cabbage, black rice). Berries such as blueberry, strawberry, cherry, blackcurrant and lingonberry are the most common sources of anthocyanins. A single serving of berries contains several hundred milligrams of anthocyanins/100 g fresh weight; for example, elderberries contain 664–1816, chokeberries 410–1480, bilberries 300–698, raspberries 20–687, blackcurrants 130–476, blackberries 82.5–325.9, and blueberries 61.8–299.6 mg anthocyanins (de Pascual-Teresa & Sanchez-Ballesta, 2007; Krga & Milenkovic, 2019).

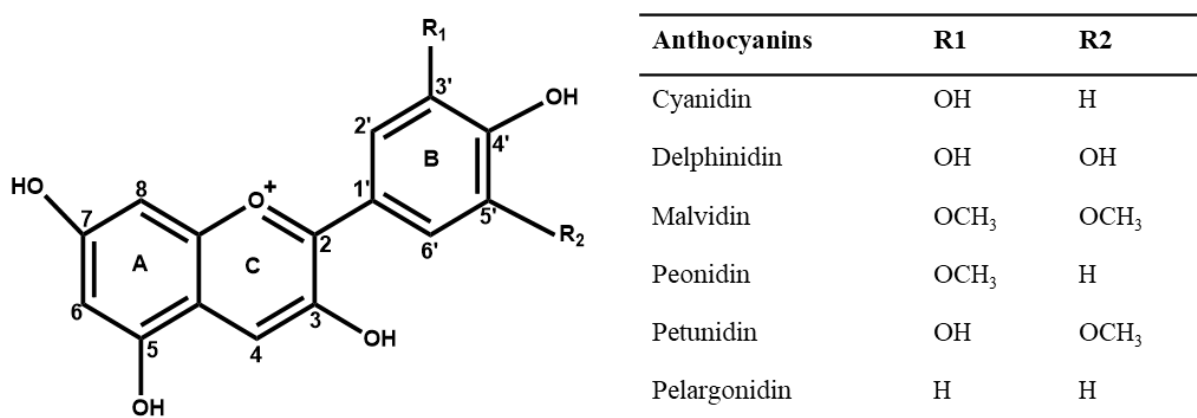


Figure 1.1 Basic structure of anthocyanins

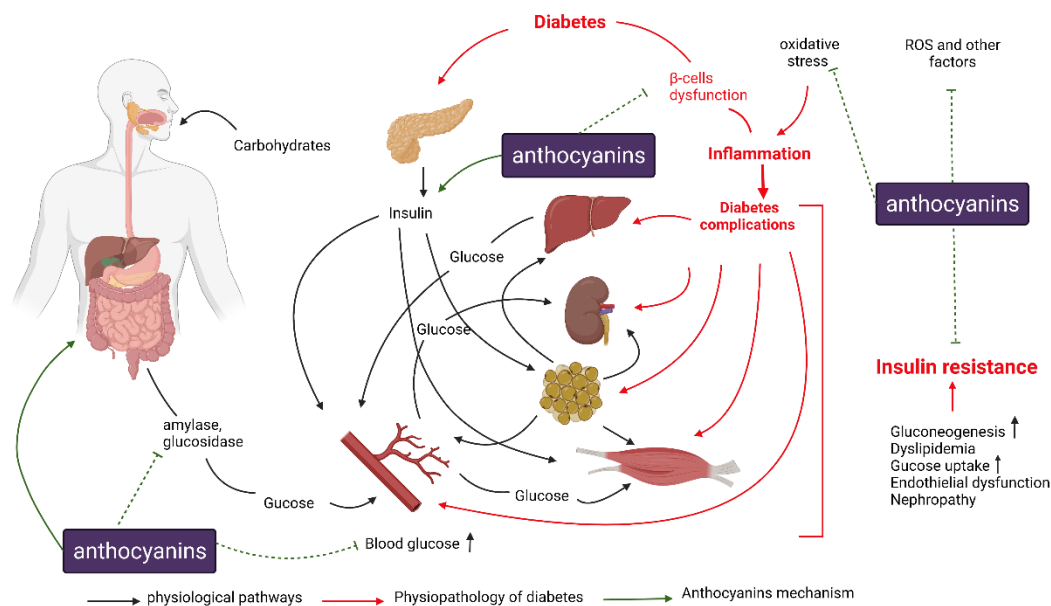
Genetic, environmental, and agronomic factors (light, temperature, humidity, fertilization, processing, and storage conditions) may influence the anthocyanin concentrations in foods considerably (Krga & Milenkovic, 2019). For example, under different processing conditions, whilst processing led to a loss of anthocyanins, blueberry anthocyanins were better preserved in canned fruits (around 72%) and purees (52%) than in clarified juice (41%) (Howard et al., 2012). Similarly, anthocyanin contents in fresh blueberry juice are higher compared to those stored for six months at 4 °C (about 10% loss) or 25 °C (about 50% loss) (Howard et al., 2016).

The dietary habits vary greatly within a population and impact on the daily anthocyanin consumption. Dietary anthocyanin intake is not well established, mainly because of the absence of available information in food-composition databases and variations in results depending on the dietary assessment used. In Europe, the estimated average intake range from 64.9 mg/day (Italy) to 19.8 mg/day (Netherlands) for men, and from 44.1 mg/day (Italy) to 18.4 mg/day (Spain) for women. In the United States, women consume on average 12.6 mg anthocyanins per day while men consume on average 10.5 mg/day. Around 50% of Europe's estimated habitual intake consists of fruits, such as grapes, apples, pears, and berries, while around 21% comes from red wine. Anthocyanin intake in the United States is primarily derived from berries (20%), wine (16%), and grapes (11%) (Wallace & Giusti, 2015).

## **1.7 Beneficial effects of anthocyanins towards diabetes**

Anthocyanins have demonstrated a range of biological properties which have been associated with the health-beneficial effects of anthocyanins towards preventing/managing T2DM. *In vitro* and *in vivo* studies have suggested that anthocyanins may lower blood glucose by improving insulin resistance, protecting  $\beta$ -cells, increasing the secretion of insulin, reducing oxidative stress and inflammation, upregulating expression of glycolytic enzymes, downregulating gluconeogenesis related gene expression and activating AMP-activated

protein kinase (AMPK). A summary of mechanisms by which anthocyanins can modulate the risk factors associated with T2DM are shown in **Error! Reference source not found.**



**Figure 1.2** The different mechanisms responsible for protective role of anthocyanins in preventing and managing T2DM (Les et al., 2021).

### 1.7.1 Role of anthocyanins in IR

The decline in the sensitivity of peripheral cells (muscle, adipose, liver) to insulin contributes to the progression and development of T2DM. Several mechanisms associated with improper enzymatic or hormonal functioning may contribute to IR. The presence of excessive visceral fat in the body is associated with IR, which results in dysregulation of carbohydrate metabolism, a decrease in insulin sensitivity of tissues, hyperglycaemia, and inflammation, and, consequently, a higher risk of T2DM (Sartipy & Loskutoff, 2003). Adipocytes of visceral fat are metabolically active and secrete adipocytokines such as adiponectin which decrease in concentration under IR, and proinflammatory cytokines such as TNF- $\alpha$  (tumor necrosis factor) or IL-6 (interleukin-6). The increased expression levels of these inflammatory molecules in T2DM is likely to contribute to the development of IR (Al-Goblan et al., 2014). Anthocyanins have shown anti-inflammatory properties and reduced the expression of TNF- $\alpha$ , MCP-1 and IL-6, contributing to the improvement of T2DM (Sasaki et al., 2007; DeFuria et al., 2009; Edirisinghe et al., 2011).

Tsuda et al. (2003) has found improved IR in mice (C57BL/6J) fed a high-fat diet enriched with anthocyanins. It was observed that diet supplemented with cyanidin-3-glucoside reduced the blood glucose and TNF- $\alpha$  expression in mice (Tsuda et al., 2003). In another study, KK-A<sup>y</sup> mice fed a diet supplemented with cyanidin-3-glucoside showed reduced glycaemia and decreased expression of inflammatory markers such as TNF- $\alpha$  and MCP-1 in adipose tissue (Sasaki et al., 2007). A high-fat diet supplemented with tart cherry powder led to decreased fasting glycaemia and insulinaemia in Zucker rats, as well as a decrease in plasma levels of IL-6 and TNF- $\alpha$  which led to improved IR (Seymour et al., 2008). Another study showed that rats fed a high-fat diet supplemented with freeze-dried whole blueberry powder had reduced levels of fasting glucose and TNF- $\alpha$  expression in adipose tissue, which are associated with improved insulin tolerance. In another study, C57BL/6 mice fed a high-fat diet supplemented with freeze-dried whole blueberry powder showed reduced levels of fasting glucose and TNF- $\alpha$  expression in adipose tissue and improved insulin tolerance test (ITT) results (DeFuria et al., 2009). The hypoglycaemic properties attribute to anthocyanins can be associated with activation of insulin receptors. For example, anthocyanins extracted from black soybeans induced insulin receptor autophosphorylation in Sprague-Dawley rats, thus increasing their activity (Nizamutdinova et al., 2009).

Anthocyanins may also exert beneficial antidiabetic effects by modulating the activity of genes involved in insulin-glucose signalling pathways. GLUT4 is the insulin-responsive transporter, mainly found in muscles and adipose tissues and responsible for postprandial glucose clearance. GLUT4 is found in intracellular vesicles in an unstimulated state. After a meal, blood glucose stimulates the insulin secretion from pancreas. In response to insulin binding to its receptor, GLUT4 transporter is translocated to the plasma membrane, thereby allowing glucose uptake, and thus maintaining glucose homeostasis (Bryant et al., 2002). Therefore, GLUT4 plays an important role in the pathophysiology of T2DM. The lack of GLUT4 expression or translocation to the plasma membrane in T2DM patients prevents insulin-dependent glucose uptake into target cells. In animal models, anthocyanins from

different plant species resulted in decreased glycaemia and an increase in GLUT4 expression, which contributes to decreased IR (Sasaki et al., 2007; Nizamutdinova et al., 2009; Chen et al., 2019). It has been found that cyanidin-3-glucoside extracted from black beans improved the IR in 3T3-L1 adipocytes through up-regulation of GLUT4 gene expression (Inaguma et al., 2011). Recently, Chen et al. (2019) has observed enhanced glucose uptake in L6 rat skeletal muscle cells after treatment with anthocyanin-rich black soybean seed coat extract. Cyanidin-3-glucoside, present abundantly in the soybean seed coat, has demonstrated the ability to upregulate Akt/GLUT4 signalling, which can decrease risk factors associated with diabetes (Chen et al., 2019).

Retinol binding protein 4 (RBP4) is, a transporter protein synthesized predominantly in liver, responsible for transport of retinol from liver to target tissues. In IR, the elevated level of RBP4 expression causes insulin signalling in the skeletal muscles to be impaired, consequently causing hepatic gluconeogenesis and elevated blood glucose levels (Graham et al., 2006). It was found that administration of dietary cyanidin to diabetic mice is associated with increased GLUT4 expression in white adipose tissue and reduced glucose 6-phosphatase (G6Pase) levels. It also decreased the levels of RBP4 in white adipose tissue and in the blood, resulting in improved glucose uptake, plasma insulin levels, and suppression of glucose production (Sasaki et al., 2007).

The AMPK pathway is considered a potential therapeutic target for T2DM because of its role in regulating glucose uptake. As AMP-activated protein kinase (AMPK) is activated in adipose tissue and skeletal muscle, GLUT4 is expressed, which augments glucose uptake and utilization by these tissues through mechanisms independent of insulin. The activation of AMPK reduced glucose production in the liver, which increases in T2DM (Viollet et al., 2009). It was found that addition of bilberry extract to the diet decreased blood glucose levels and increased insulin response in diabetic mice through activation of AMPK in adipose, muscle, and liver tissue (Takikawa et al., 2010). As a consequence, anthocyanins may modulate adipokine expression, increase GLUT4 expression, decrease RBP4 expression,

activate AMPK and reduce oxidative stress, thereby enhancing insulin sensitivity and affecting glycaemic control.

### **1.7.2 Role of anthocyanins in pancreatic $\beta$ -cell protection**

T2DM diabetes is associated with the impairment of pancreatic  $\beta$ -cell function and insulin secretion. The persisting hyperglycaemic conditions result in increased intracellular reactive oxygen species (ROS) production which has been shown to lead to pancreatic  $\beta$ -cell apoptosis (Fu et al., 2013). Anthocyanins have demonstrated protection towards  $\beta$ -cell function through the modulation of antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase present in pancreatic islets (Roy et al., 2008; Nizamutdinova et al., 2009; Zhang et al., 2011; Hong et al., 2013). Low expression levels of these antioxidant enzymes have been shown to increase susceptibility of  $\beta$ -cells to oxidative stress caused by high glucose conditions (Lenzen, 2008; Drews et al., 2010). The administration of different anthocyanin-rich extracts such as cornelian cherries and black soya bean in streptozotocin (STZ) or alloxan (ALX)-induced diabetic rodents fed with normal diet or high fat diet (HFD) have demonstrated improved insulin secretion and  $\beta$ -cell function in these animals (Jayaprakasam et al., 2006; Nizamutdinova et al., 2009). *In vitro* studies also indicated the protective role of anthocyanins such as from purple corn towards improving diabetes symptoms, but the exact mechanism is not understood (Hong et al., 2013). Further studies are therefore needed in order to determine the exact mechanism through which anthocyanins affect  $\beta$ -cell function/dysfunction, insulin secretion, and therefore glucose metabolism.

### **1.7.3 Inhibition of carbohydrate digesting enzymes**

Since inhibition of the carbohydrate digestive enzymes such as  $\alpha$ -amylases and  $\alpha$ -glucosidases reduces the postprandial glycaemia, it is considered an important approach to counteract metabolic alterations related to hyperglycaemia and T2DM. Currently, synthetic enzymes inhibitors, such as acarbose and miglitol, are widely used as antidiabetic drugs. Due to the adverse effects of synthetic inhibitory drugs, interest has increased in finding novel

natural agents that can successfully inhibit carbohydrate hydrolysing enzymes. Many studies have demonstrated that anthocyanin-rich diet may deliver glucomodulatory effects by inhibiting starch digestion enzymes and managing the postprandial hyperglycaemia. For example, anthocyanins from black carrot inhibited the  $\alpha$ -amylase,  $\alpha$ -glucosidase, and DPP-IV enzymes with  $IC_{50}$  values lower than synthetic inhibitors such as acarbose and vildagliptin. The cyanidin 3-xylosyl galactoside found in black carrot is considered the most predominant anthocyanin for enzyme inhibition (Karkute et al., 2018). Anthocyanin rich fruits such as blackcurrant, blueberry, chokeberry, cranberry, lingonberry, sweet cherry and strawberry have shown similar effects (Podsdek et al., 2014). McDougall et al. (2005) has found blueberry and blackcurrant as potent inhibitors of rat intestinal  $\alpha$ -glucosidase with  $IC_{50}$  of 18 and 22.5  $\mu$ g phenol/assay, respectively. Red berries such as raspberry and strawberry contain anthocyanins such as cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, and peonidin-3-O-glucoside which showed inhibitory potential against carbohydrate digestive enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase) (Zhang et al., 2010; Gutierrez-Albanchez et al., 2019). Cyanidin and cyanidin-3,5-diglucoside have been identified in grape extract and demonstrated competitive inhibitory effects against  $\alpha$ -glucosidase (You et al., 2011). Only a small number of studies have investigated purified anthocyanins against digestive enzymes. For instance, cyanidin and its derivatives such as cyanidin-3-O-rutinoside, cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, and peonidin-3-O-glucoside were identified as effective  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors (Adisakwattana et al., 2004; Adisakwattana et al., 2012; Sui et al., 2016). Different fruit extracts have different inhibitory properties against starch digestion enzymes. Blackberry, black currant and Aronia berry are potent  $\alpha$ -amylase inhibitors compared to other fruits measured by the same method (Podsdek et al., 2014; Ho et al., 2017; Tan & Chang, 2017). In the case of  $\alpha$ -glucosidase, blueberry and blackcurrant were the most effective inhibitors followed by strawberry, raspberry, and red cabbage (McDougall et al., 2005). Even for the same fruit, different studies reported different inhibitory potential. Different factors, such as type and dose of extract, method of determination, type and concentration of enzyme and substrate can contribute to variable results as indicated in

a recent systematic review investigating the inhibitory potential of polyphenol-rich fruits (Prpa et al., 2021).

The above-mentioned studies suggested that anthocyanins can inhibit both  $\alpha$ -glucosidase and  $\alpha$ -amylase. However, the results are inconsistent, for example, Ostberg-Potthoff et al. (2019) have reported that both anthocyanin-rich and ellagic acid derivative-rich pomegranate extracts inhibited  $\alpha$ -amylase in the similar manner, with  $IC_{50}$   $1141 \pm 93$  mg/mL and  $1163 \pm 42$  mg/mL, respectively. In contrast, Bellesia et al. (2015) have found that a pomegranate extract rich in anthocyanins has a lower  $\alpha$ -amylase inhibitory activity than one rich in ellagitannins. McDougall et al. (2005) also showed that anthocyanin-rich raspberry extract inhibited  $\alpha$ -amylase less efficiently than an ellagitannin-rich raspberry extract (28% and 75% inhibition, respectively, at 50 mg phenol/assay). In the same study, it was reported that anthocyanin-rich raspberry extract inhibited  $\alpha$ -glucosidase (82% at 50  $\mu$ g/mL) more efficiently than the crude extract (32% at 50  $\mu$ g/mL) (McDougall et al., 2005). Recently, Ho et al. (2017), also found that ellagitannin-rich cloudberry extract showed greater inhibition of  $\alpha$ -amylase than anthocyanin-rich blueberry extract. It was demonstrated that anthocyanin-rich extracts were more potent  $\alpha$ -glucosidase inhibitors compared to  $\alpha$ -amylase (Ho et al., 2017). Recently, different fractions of Aronia berry extract investigated for enzyme inhibition demonstrated stronger inhibition of  $\alpha$ -amylase in the presence of polyphenol-rich fraction ( $IC_{50}$   $123 \pm 8$  mg/mL) compared to anthocyanin-rich extract ( $IC_{50}$   $677 \pm 63$  mg/mL) (Ostberg-Potthoff et al., 2019). In the same study, it was reported that an anthocyanin-rich extract of pomegranate was more effective at  $\alpha$ -glucosidase inhibition ( $IC_{50}$   $57.2 \pm 6.4$  mg/mL) compared to ellagitannin-rich ( $IC_{50}$   $169 \pm 22$  mg/mL) and polymeric polyphenol-rich pomegranate fractions ( $IC_{50}$   $116 \pm 3$  mg/mL) (Ostberg-Potthoff et al., 2019). However, cloudberry extracts with high ellagitannin content did not inhibit  $\alpha$ -amylase as effectively as raspberry extracts (Grussu et al., 2011); ellagitannins which are dominant in both cloudberry and raspberry fruits are sanguin H-6 and lambertian C (McDougall et al., 2008). The authors proposed that anthocyanins enhanced  $\alpha$ -amylase inhibition by ellagitannins (Grussu

et al., 2011). Overall, studies were consistent in demonstrating inhibition of  $\alpha$ -amylase enzyme by polyphenolic and/or ellagitannin-rich fruit extracts in contrast to  $\alpha$ -glucosidase, which was found more sensitive to anthocyanin contents of fruit extracts.

However, (Akkarachiyasit et al., 2010) has been found that Cyanidin-3-rutinoside was stronger amylase inhibitor ( $IC_{50}$   $24.4 \pm 0.1 \mu M$ ) than maltase ( $IC_{50}$   $2323 \pm 14.8 \mu M$ ) and sucrase ( $IC_{50}$   $250.2 \pm 8.1 \mu M$ ). Recently, anthocyanin-rich fruits strongly inhibited  $\alpha$ -glucosidase compared to  $\alpha$ -amylase. However, very low inhibition (%) was determined for individual anthocyanins and copigments for both  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Berger et al., 2020). Despite the fact that *in vitro* studies demonstrated anti-diabetic properties of anthocyanins, there are some limitations to comparing these outcomes directly against each other, including the method of detection, concentrations of anthocyanins, and enzyme/substrate sources

## **1.8 Mode of carbohydrate digesting enzyme inhibition**

The most prominent mechanism responsible for antidiabetic effects of anthocyanins in controlling the postprandial glycaemia is through inhibition of digestive enzymes which has been described as competitive or mixed type. It was proposed that glucosyl groups associated with anthocyanins might have structural similarities to normal substrates of  $\alpha$ -glucosidase, which may be able to bind to active sites of enzymes without hydrolysing them (McDougall & Stewart, 2005). Another possible mechanism could be that the hydroxyl groups on anthocyanins may also interact with polar groups in the enzyme's active site, thereby changing its molecular composition and properties, and hence affecting its activity (Adisakwattana et al., 2009). The inhibitory potential of anthocyanins is linked to their chemical structure and type and/position of sugar moiety attached. Adding a hydroxyl group (-OH) in the flavonoid skeleton at 5- and 7-positions of the A-ring, at 3' and 4'- positions of the B-ring and at 3-position of the C-ring, and the C2=C3 double bond in the C-ring, were crucial for the inhibition behaviour of flavonoids (Proença et al., 2017; Martinez-Gonzalez et al., 2019). It has been found that delphinidin (three hydroxyl groups on the B ring) inhibited

the  $\alpha$ -glucosidase more efficiently than cyanidin and petunidin which have two hydroxyl moieties (Promyos et al., 2020). The hydroxyl moieties at C-3' and C-4' of the B ring of cyanidin exhibited higher inhibitory activity than hydroxyl moieties at the C-4' and C-5' of the B ring of petunidin (Promyos et al., 2020).

The glycosylation of anthocyanins influences the inhibitory potential. It was proposed that suitable glucoside substitutions could increase the number of total aromatic hydroxyl groups consequently encouraging enzyme inhibitory activities. For instance, the hydroxy groups at C-3 position of ring C, C-3' and C-4' position of ring B, and the glucoside substitutions at the C-3 position of ring C were crucial for the enzyme inhibition activities of flavanols. The previous study showed that cyanidin exhibited an  $IC_{50}$  value higher than cyanidin-3-glucoside (Akkarachiyasit et al., 2010). Nevertheless, glycosylation and the substituting of different sugars could reduce the  $\alpha$  glucosidase inhibiting capacity of anthocyanins owing to lack of specific A- and B-ring hydroxyl groups in the right stereospecific orientation to interact successfully with the catalytic site of the enzyme (Xu et al., 2019). The inhibitory activity was decreased to a greater extent with a larger number of glycoside substitutions on flavonoids , for example, cyanidin-3-glucoside demonstrated more inhibitory activity compared to cyanidin-3,5-diglucoside (Akkarachiyasit et al., 2010). A previous study showed that inhibitory potential of flavonoids was decreased to a greater extent with a larger number of glycoside substitutions (Kim et al., 2000).

## **1.9 Anthocyanins and postprandial glycaemia**

The above-mentioned antidiabetic effects of anthocyanins have demonstrated the potential of anthocyanins/anthocyanin-rich foods to prevent and manage T2DM. Animal and human studies have demonstrated the beneficial effects of anthocyanin consumption in the regulation of postprandial glycaemia. Anthocyanins (1 g of anthocyanins/1 kg high-fat diet), extracted from Cornelian cherries improved glucose tolerance in mice fed on a high-fat diet (60% energy from fats) supplemented with Cornelian cherries extract. Castro-Acosta et al. (2016) investigated the influence of anthocyanin-rich blackcurrants drink on postprandial

glucose response in humans. It was found that a blackcurrant drink containing 600 mg anthocyanins reduced the postprandial glycaemia and insulinaemia following the consumption of a starch- and sucrose-rich meal. Attenuated and/or delayed postprandial blood glucose concentrations have been observed in healthy adults following anthocyanin-rich berries mixture (bilberries, blackcurrants, cranberries, strawberries) consumed with sucrose (Torrönen et al., 2010). The consumption of green tea mixed with freeze-dried apple peel, blackberry, blackcurrant and strawberry powders significantly lowered the postprandial glucose and insulin response in healthy volunteers (Nyambe-Silavwe & Williamson, 2016). Reduced plasma glucose peak has been observed following maqui berry extract (containing  $\geq 25\%$  delphinidins and  $\geq 35\%$  total anthocyanins) in glucose intolerant volunteers (Hidalgo et al., 2014). In contrast, the positive effects of anthocyanin-containing foods on glycaemic response have not been duplicated in other studies of this nature. For example, pancakes with raspberries and blueberries do not affect glycaemic response compared to a control in healthy subjects (Clegg et al., 2011). A direct comparison is not possible due to differences in the methodological approaches used in these studies, for example, anthocyanin content, carbohydrate source, and study design. Despite not being consistent, the results suggest that anthocyanins/anthocyanin-rich foods may modulate postprandial glycaemic response and prevent/manage T2DM.

### **1.10 *Hibiscus sabdariffa***

*Hibiscus sabdariffa* (HS), commonly known as "red sorrel" or "roselle", belongs to the malvaceae family. HS contains many bioactive molecules, among them the most important are anthocyanins. Previous studies have found delphinidin-3-sambubioside and cyanidin-3-sambubioside as major anthocyanins found in HS (Sindi et al., 2014; Ifie et al., 2016). Recently, the flower or calyces of this plant gained much attention in experimental and clinical studies due to its anthocyanin rich profile and cardioprotective properties (Gurrola-Diaz et al., 2010; Chang et al., 2014).

Several *in vitro* studies on different cell lines such as rat hepatocytes, mouse macrophages (RAW 264.7 and J774A.1) and preadipocytes 3T3-L1 and human leukemia cells HL- 60 have demonstrated the antioxidant potential of HS (Table 1.1). Dried flower extracts of HS at a dose level of 0.10 and 0.20 mg/mL were effective in preventing the rat cells from t-BHP induced cytotoxicity and genotoxicity through free radical scavenging and preventing unscheduled DNA synthesis (Tseng et al., 1997). HS anthocyanins inhibited the oxidation of low density lipoproteins (LDL) and reduced the CD36 gene expression and thus possess the antiatherosclerotic capacity (Kao et al., 2009). Similar results were reported by Chang et al. (2006).

The results from animal studies (Table 1.2) have indicated antioxidant (Ali et al., 2003) hypoglycaemic (Sachdewa & Khemani, 2003; Peng et al., 2011) and lipid lowering effects of HS (Hirunpanich et al., 2006; Farombi & Ige, 2007; Yang et al., 2010). It was demonstrated that the blood glucose-lowering effects of HS at a dose of 200 mg/kg was comparable to the therapeutic effect of lovastatin in diabetic rats (Farombi & Ige, 2007). HS polyphenolic extracts enhanced activity of hepatic fatty acid, lipoprotein enzymes and receptors, and renal function, which improved lipid profile (Lee et al., 2009; Yang et al., 2010).

The clinical studies conducted in pre- and mildly hypertensive (Mozaffari-Khosravi et al., 2009; McKay et al., 2010) to stage 1 or 2 hypertensive subjects (Haji Faraji & Haji Tarkhani, 1999; Herrera-Arellano et al., 2007) have demonstrated the hypotensive and hypolipidemic activities of HS (Table 1.3). HS administration reduced the serum cholesterol (9-12 %), triglycerides (9.9%) and high density lipoproteins (8.3%) in subjects with poor lipid metabolism (Lin et al., 2007; Hajifaraji et al., 2018). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) decreased with HS treatment in most of these studies. A current systematic review and meta-analysis has also confirmed the blood pressure lowering effects of HS in humans (Ellis et al., 2021). The health benefits of the HS consumption might be related to its high polyphenolic profile, in particular, anthocyanins. More attention has focused recently on increasing the knowledge about the HS plant as a source of functional

food. Despite the fact that some animal studies and *in vitro* studies (Table 1.4 & Table 1.5) have suggested the antidiabetic properties of HS, *in vivo* human studies are still lacking. Therefore, HS was used as a source of anthocyanins to investigate the glucose lowering effects *in vivo* and *in vitro*.

**Table 1.1 Summary of cell culture studies showing different effects of *Hibiscus sabdariffa***

Cell	Type of extract	Treatment	Mode of action	Related effects	References
Mouse macrophages cell line RAW264.7	Anthocyanins	1, 1.2, and 2 mg/mL	Antioxidant Antiapoptotic capacity	↓LDL oxidation ↓macrophage death	Chang et al. (2006)
Mouse macrophages cell line J774A.1	Anthocyanins rich extract	0.05 – 2 mg/ mL	Antiatherosclerotic Inhibition of LDL oxidation	↓LDL oxidation ↓PPAR-gamma ↓LDL CD36 ↓oxidized LDL absorption by macrophages	Kao et al. (2009)
Preadipocytes 3T3-L1		0, 250, 1000, 2000, and 5000 µg/mL 5 days	Inhibition of adipocyte differentiation	↓lipid accumulation ↓ PPAR-δ ↓C/EBPα	Kim et al. (2007)
Human leukaemia cells (HL-60)	PCA	0.2-2 mM 24-48h	Antitumoral Antioxidant	↓phosphorylation ↓expression of Bcl-2 protooncogene	Tseng et al. (2000)
Rat hepatocytes	PCA	0.05 and 0.10 mg/mL (1.5 mM t-BHP for 30 min to induce cell damage)	Antioxidant		Tseng et al. (1996)
Rat hepatocytes	PCA	0.1, 0.2, 0.5, and 1mg/mL (1.5 mM t-BHP for 30 min to induce cell damage)	Antioxidant	↑radical scavenging ↓LDH leakage ↓MDA formation	Tseng et al. (1997)

**Abbreviations:** LDL (low density lipoproteins); PPAR-gamma (peroxisome proliferator-activated receptor); CD36 (cluster of differentiation 36); PCA (protocatechuic acid); C/EBPα (CCAAt-enhancer-binding protein); t-BHP (tert-Butyl hydroperoxide); MDA (malonaldehyde); ↓ indicates a decrease; ↑ indicates an increase.

**Table 1.2 Summary of animal studies showing different health effects of *Hibiscus sabdariffa***

Animal model	Study design	Treatment	Duration (weeks)	Mode of action	Related effects	References
Rats (36)	Case-control	Aqueous extract 50, 100, and 200 mg/kg anthocyanins Hepatotoxicity induced by 700 mg/kg acetaminophen	2, 3, or 4 5 days	Antioxidant	High dose of anthocyanins improves liver function	Ali et al. (2003)
Rats hypercholesterolemia induced through diet	Case-control	G 1: baseline diet G 2, 3, and 4: experimental diet with 5, 10, and 15 g HS/100 g of diet, respectively	4	Antiobesity and lipid lowering	↓increased weight in G3 and G4 ↓TC and LDL in all groups	Carvajal-Zarrabal et al. (2005)
Rabbits (30)	Case-control	G1: control G2: HCD G3:1% HS G4: HCD+0.5% HS G5: HCD+1% HS	10	Lipid lowering and antiatherosclerotic	↓TC, CHOL, and LDL in G4 and G5 ↓foam cell formation in blood vessels	Chen et al. (2003)
Rats (Diabetic)	Case-control	250 mg/kg BW	3	Hypoglycaemic	↓glucose (16-26%), ↑insulin (10-14%) ↓TG (30%)	Sachdewaand Khemani (2003)
Male albino mice (30) Alloxan-induced diabetes	Case-control	100 and 200 mg/kg BW of HS versus lovastatin (10 mg/kg)	4	Lipid lowering and antioxidant	↓LDL and CHOL at dose 200 mg/kg	Farombiand Ige (2007)
SD Rats (42) (Hypercholesterolemic)	Case-control	250, 500, and 1000 mg/ kg BW of HS intragastrically	6	Hypolipidemic	At doses 500 and 1000 mg/kg ↓serum CHOL (22 and 26%) ↓LDL (22 and 32%) ↓TG (33 and 28%)	Hirunpanich et al. (2006)
Hamsters	Case-control	G1: control G2: HFD G3: HFD+0.5% HS G4: HFD+1% HS G5: HFD+2% HS	10	Anti-obesity	↓TG, CHOL, LDL ↓liver CHOL ↓ALT and AST ↓adipocytes differentiation ↓fatty acid synthesis	Kao et al. (2016)
Hamsters	Case-control	G1: control G2: HFD (10% coconut oil+0.5% cholesterol) G3: HFD+25 mg G4: HFD+50 mg G5: HFD+100 mg G6: HFD+ 25 mg anthocyanins	10	Anti-obesity and hepatoprotective	↓weight gain in G5 ↓fatty liver (dose dependent) ↑PON-1para activity ↓AST and ALT	Huang et al. (2015)
Rats (68) (Diabetics)	Case-control	G1: control G2: HPE (normal diet+200 mg/kg HPE) G3: HFD G4: HFD+HPE (100mg/kg) G5: HFD +HPE (200mg/kg) G6: HFD+STZ G7: HFD+STZ+ HPE (100mg/kg) G8: HFD+STZ+ HPE (200mg/kg)	7	Hypoglycaemic and hypoinsulinemic	↓glucose (60-65%) in G5 ↓ elevation in AGE ↓CTGF and RAGE	Peng et al. (2011)
<b>Hamsters</b>		G1: control G2: HFD G3: HFD+HS (1-2% w/w)	10	Hypolipidemic and hepatoprotective	↓LDL (60%) in G4 as compared to G3 (45%) ↓serum LDL and CHOL Activation of AMPK	Yang et al. (2010)

---

G4: HFD+HPE (0.1-0.2% w/w)

↓SREBP-1  
↓ expression of fatty acid synthase

---

Abbreviation: G1, G2, G3 (groups); HS (Hibiscus sabdariffa); TG (total glycerides); TC (total cholesterol); LDL (low density lipoproteins); CHOL (cholesterol); BW (body weight); HCD (high cholesterol diet); HFD (high fat diet); ALT (alanine aminotransferase); AST (Aspartate transaminase); PON (paraonase); STZ (streptozocin); CTGF (connective tissue growth factor); AGE (advanced glycation end product); AMPK (adenosine monophosphate-activated protein kinase); SREBP-1 (Sterol regulatory element-binding protein 1); RAGE (receptor of AGE)

**Table 1.3 Summary of human intervention studies showing different health effects of *Hibiscus sabdariffa***

Design	Population	Size	Duration (weeks)	Dose/day	Related effects	References
Randomized controlled G1: black tea (BT) G2: sour tea (ST)	Type 2 Diabetic mildly hypertensive	60	4	2 g/240 mL 2 times	↓MSBP in G2 (16%), ↑MSBP in G1 (7%) No significant change on MDBP in either group ↓MPP in G2 (33%), ↑MPP in G1 (13%)	Mozaffari-Khosravi et al. (2009)
Randomized, double blind, placebo-controlled G1: placebo (ST) G2: ST	Pre- & mildly hypertensive	65	6	1.25 g/240 mL 3 times	↓MSBP in G2 (5.5%), ↓MSBP in G1 (2.4%) No significant change on MDBP in either group ↓MAP in G2 (4.6%), ↑MAP in G1 (0.9%)	McKay et al. (2010)
Randomized, double blind, placebo-controlled G1: placebo (blackcurrant) G2: GT (240mg catechins) G3: HS (250 mg anthocyanins)	Healthy volunteers	54	6	450 mg powered tablet containing 250 mg anthocyanins 1 time with lunch	↓SBP in G3 (8.1%) No significant change in DBP ↓TC, LDL, TAC, and ↑HDL in three group	Kafeshani et al. (2017)
Randomized, controlled G1: HS infusion G2: 2 tablets captopril (25 mg)	Mild to moderate hypertensive	75	4	10 g HS (9.6 mg anthocyanins) 1 time before breakfast	HS is more effective against BP as compared to captopril	Herrera-Arellano et al. (2004)
Randomized, controlled G1: HS infusion G2: lisinopril (10 mg)	Hypertensive	193	4	Dose providing 250 mg anthocyanins	HS is less effective against BP as compared to drug	Herrera-Arellano et al. (2007)
Randomized, cross over G1: 1 capsule G2: 2 capsules G3: 3 capsules	Elevated cholesterol level (175 to 327 mg/dL)	42	4	500 mg capsule (powder) 3 times	↓serum CHOL (12%)	Lin et al. (2007)
Factorial, randomized, follow up G1: non MeSy G2: MeSy	Persons with Non MeSy and MeSy syndrome	222	4	100 mg capsule 1 time	↓glucose, ↓CHOL, ↑HDL in G2 ↓TG in both groups	Gurrola-Diaz et al. (2010)
Randomized, controlled G1: control (BT) G2: intervention (ST)	Persons with polygenic dyslipidaemia	43	12	2 g/240 mL 2 times	↓TC 99.46%, ↓HDL-C (8.33%), ↓LDL-C (9.80%), ↓TG (9.90%)	Hajifaraji et al. (2018)
Randomized, double blind G1: control (500 mg starch) G2: HS treated (450 mg HS+50 mg starch)	Fatty liver	36	12	1 capsule 3 times	↓BW, ↓BMI, ↓FS	Chang et al. (2014)
Randomized, open label, two way cross over G1: HS G2: tap water	Healthy volunteers	8	Acute	10 g/200 mL Single dose	↑AO potential in plasma and urine, ↓MDA	Frank et al. (2012)

**Abbreviation:** G1, G2, G3 (groups); BT (black tea); ST (sour tea); HS (*Hibiscus sabdariffa*); MSBP (mean systolic blood pressure); MDBP (mean diastolic blood pressure); MPP (mean pulse pressure); MAP (mean arterial pressure); MeSy (metabolic syndrome); TG (triglycerides); TC (total cholesterol); TAC (total acylglycerol) LDL (low density lipoproteins); HDL (high density lipoprotein); CHOL (cholesterol); MDA (malonaldehyde); BW (body weight); BMI (body mass index); FS (fatty liver score); A0 (antioxidant)

**Table 1.4 *In vitro*  $\alpha$ -glucosidase inhibition by *Hibiscus sabdariffa***

Method of detection	Inhibitor	Substrate (source)	IC <sub>50</sub> glucosidase (yeast/rat)	IC <sub>50</sub> rat intestinal maltase	IC <sub>50</sub> rat intestinal sucrase	Buffer (pH)	Incubation time	References
<b>detection of chromophore released from synthetic substrate</b>	Aqueous extract	<i>p</i> NPG	433.93 $\mu$ g/mL	-	-	0.1 M phosphate (6.9)	5 min 25 °C	Alegbe et al. (2019)
	Protocatechuic acid		24.30 $\mu$ g/mL	-	-			
	Gallic acid		22.29 $\mu$ g/mL	-	-			
<b>detection of chromophore released from synthetic substrate</b>	different cultivars of hibiscus	<i>p</i> NPG	627 and 723 $\mu$ g/mL	-	-	100 mM potassium phosphate (7)	30 min at 37 °C	Rasheed et al. (2018)
	Acarbose		1415 $\mu$ g/mL (2.19 mM)	-	-		30 min at 37°C	
<b>detection of chromophore released from synthetic substrate</b>	ethanol extract	<i>p</i> NPG	$\alpha$ -glucosidase 15.81 $\mu$ g/mL	-	-	Phosphate (7)	30 min at 37 °C	Gondokesumo et al. (2017)
	ethanol extract		$\beta$ -glucosidase 41.77 $\mu$ g/mL	-	-		15 min at 37 °C	
	Acarbose		$\alpha$ -glucosidase 9.45 $\mu$ g/mL	-	-		30 min at 37 °C	
	Acarbose		$\beta$ -glucosidase 22.57 $\mu$ g/mL	-	-		15 min at 37 °C	
<b>glucose oxidase method</b>	dried powder aqueous extract	Maltose 3mM	-	4.35 - 5.9 mg/mL	-	10 mM phosphate (7)	30 min at 37 °C	Ifie et al. (2016)
<b>detection of chromophore released from synthetic substrate</b>	Aqueous ethanol extract Reconstituted in DMSO and further diluted with buffer	<i>p</i> NPG	203.3 $\mu$ g/mL	-	-	100 mM potassium phosphate buffer (6.8)	10 min 25 °C	Hamza et al. (2015)
<b>detection of chromophore released from synthetic substrate</b>	Powder extract reconstituted in DW and further diluted with buffer Acarbose	<i>p</i> NPG	25.2 $\mu$ g/mL (rat) 3.5 $\mu$ g/mL (rat)	-	-	0.1M phosphate buffer (6.9)	5 min 25 °C	Ademiluyiand Obboh (2013)
<b>glucose oxidase method</b>	spray dried powder aqueous extract	86 mM Maltose 400 mM Sucrose	-	>5 mg/mL	>5 mg/mL	0.1 M phosphate (6.9)	30 min (maltase) 60 min (sucrose)	Adisakwattana et al. (2012)

**Table 1.5 *In vitro*  $\alpha$ -amylase inhibition by *Hibiscus sabdariffa***

Method of detection	Inhibitor	Substrate (source)	Enzyme	IC <sub>50</sub>	Buffer (pH)	Incubation time	References
<b>DNS method</b>	Aqueous extract Protocatechuic acid Gallic acid	Starch 1% (w/v)	PPA (0.5 mg/mL)	411.73 $\mu$ g/mL 27.03 $\mu$ g/mL 20.12 $\mu$ g/mL	0.02 M sodium phosphate buffer (pH 6.9)	10 min 25 °C	(Alegbe et al., 2019)
<b>Starch iodine method Quantification of reducing sugar (maltose equivalent) liberated from starch</b>	Ethanol extract Acarbose	Starch	$\alpha$ -Amylase from Bacillus amyloliquefaciens	18.09 $\mu$ g/mL. 3.64 $\mu$ g/mL	Phosphate (7)	15 min at 37°C	(Gondokesumo et al., 2017)
<b>DNS method</b>	Powder extract reconstituted in DW and further diluted with buffer Acarbose	Starch 1% (w/v)	PPA (0.5 mg/mL)	187.9 $\mu$ g/mL 59.8 $\mu$ g/mL	0.02M sodium phosphate buffer (pH 6.9 with	10 min at 25 °C	(Ademiluyi & Oboh, 2013)
<b>DNS method</b>	spray dried powder aqueous extract	Starch 1% (w/v)	PPA (3 units/mL)	3.52 mg/mL	0.1 M phosphate buffer saline, pH 6.9	10 min at 37 °C	(Adisakwattana et al., 2012)
<b>Reaction was stopped by adding 0.54 mL 50 % acetic acid</b>	Hibiscus acid Hibiscus acid 6 methyl ester	Corn starch	PPA (2.11 U/mL)	1.1 mM 3.22 mM	0.05 M Tris-HCl buffe buffer (pH 6.9) containing 0.01 M CaCl <sub>2</sub>	10 min at 37 °C	(Hansawasdi et al., 2000)

## 1.11 Aims and objectives

The current chapter has described the current understanding on diabetes, complications and risk factors associated with hyperglycaemia, the role of glycaemic control in the management of T2DM, dietary sources and intake of anthocyanins, and their potential antidiabetic effects and possible mechanisms. Based on *in vitro* and *in vivo* findings in the present literature, remaining research gaps have been highlighted, some of which have been addressed in the present study.

Indeed, studies have suggested that anthocyanins/anthocyanin-rich foods can modulate the postprandial hyperglycaemia and play part in the management of diabetes. Among the fruits, berries are the main source of anthocyanins and have been extensively studied for their antidiabetic effects. In addition to berries, anthocyanins are also present in some edible flowers, such as red hibiscus and roses.

Therefore, the main aim of the research project was to investigate the *in vitro* and *in vivo* effects of anthocyanins/anthocyanin-rich samples on hyperglycaemia. The objectives of this thesis are as follows:

- to establish *in vivo* postprandial response following consumption of fruit concentrate-based beverages i.e. blueberry and hibiscus
- to analyse and compare anthocyanin composition in these and other anthocyanin-rich juices
- to investigate the *in vitro* inhibitory effects of anthocyanin-rich samples on the activity of carbohydrate digestive enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase)
- to determine  $\alpha$ -amylase inhibitory properties in a range of anthocyanin-rich samples using the commonly used DNS assay
- to compare the performance of an absorbance-based kinetic assay that is utilizing a synthetic substrate, against the DNS assay
- to determine the mode of  $\alpha$ -amylase inhibition of anthocyanins/anthocyanin-rich samples

## Chapter 2

### Materials and methods

In this chapter, the general analytical methods including reagents and buffer preparation, *in vitro* enzyme inhibition assays and *in vivo* human study design that apply to more than one chapter are described.

#### 2.1 Materials

##### 2.1.1 Chemicals and reagents

Delphinidin-3-O-sambubioside (DS), cyanidin-3-O-sambubioside (CS), delphinidin-3-O-glucoside and cyanidin-3-O-glucoside were obtained from Extrasynthese, Genay, France. Folin–Ciocalteu reagent, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium carbonate, 3,5-Dinitrosalicylic acid (DNS), potassium sodium tartrate, sodium hydrogen phosphate, and sugar standards (D-glucose, D-fructose and D-sucrose), amylose (from potato starch), porcine pancreatic amylase (PPA),  $\alpha$ -glucosidase (from *Saccharomyces cerevisiae*), *p*-nitrophenyl glucopyranoside (*p*NPG), 2-chloro-4 nitrophenyl  $\alpha$ -D-maltotrioxide (CNP3), and pure compounds; quercetin, gallic acid, chlorogenic acid, protocatechuic acid and cyanidin were all purchased from Sigma-Aldrich. Co., Ltd., Dorset, UK. Acetonitrile and formic acid of LCMS grade were purchased from Fisher Scientific (Loughborough, UK). Oasis MAX cartridges (30 mg) were purchased from Waters Corporation Ltd., Milford, MA, U.S.A. Acarbose was purchased from Acros organics (Fisher Scientific Ltd., Loughborough, UK).

Hibiscus concentrate was kindly provided by IBIS Organics, Carlisle, UK. Blueberry, cherry, and pomegranate (100% fruit concentrate) were provided by Active Edge Nutrition Ltd. UK. Blackcurrant (purified anthocyanin extract, >95% purity) was provided by Meryem Benhoud, Keracol, University of Leeds (Farooque et al., 2018). Crude and purified anthocyanin containing samples of mahaleb cherry and black carrot were provided by Federica Blando, Italy (Blando et al., 2018).

### **2.1.2 Sample preparation**

Concentrated stock solutions of pure anthocyanins and purified/non-purified anthocyanins samples were made in acidified methanol (0.1% trifluoroacetic acid) solution and stored in aliquots at  $-20\text{ }^{\circ}\text{C}$ . The stock solutions of other polyphenolic compounds (gallic acid, gallic acid, chlorogenic acid, protocatechuic acid) were prepared in 10% ethanol in stored in aliquots at  $-20\text{ }^{\circ}\text{C}$ . The working dilutions for enzyme assays were prepared in respective buffer solutions. Blueberry, cherry, pomegranate and hibiscus concentrates were diluted 1:10 with Millipore water and centrifuged (5000 rpm:  $4\text{ }^{\circ}\text{C}$ ) for 5 min. The supernatants were taken and further diluted in buffer solutions to provide a range of concentrations for enzyme assays.

## **2.2 Analytical methods**

All the reagents and buffer solutions were prepared in Millipore water.

### **2.2.1 Total phenolic content assay**

#### **Folin-Ciocalteu reagent (12.5% v/v)**

Folin-Ciocalteu phenol reagent (2 N) was diluted 8 folds with water in an amber bottle prior to analysis of total phenol content.

#### **Sodium carbonate solution (4% w/v)**

Sodium carbonate anhydrous (99.9%, 40 g) was dissolved in water and diluted to 1 L in a volumetric flask. The solution was immediately covered with aluminum foil and stored at ambient temperature.

#### **Procedure**

The Folin's assay is based on the reduction of Folin–Ciocalteu reagent in the presence of phenolics resulting in the production of molybdenum–tungsten blue that is measured spectrophotometrically at 760 nm and the intensity increases linearly with the concentration of phenolics in the reaction mixture (Singleton et al., 1999).

The total polyphenol content of fruit concentrates was evaluated using the Folin-Ciocalteu method adapted to 96 well plate format (Fernando et al., 2021) with slight modifications. Briefly, 10  $\mu$ L of appropriate diluted sample was mixed with 40  $\mu$ L of Folin reagent (12.5%) and 150  $\mu$ L of sodium carbonate (4%) solution in a 96 well plate and the reaction mixture was incubated for 30 min at room temperature in the dark. Subsequently, absorbance of samples and blank was measured at 765 nm using a Tecan Spark plate reader. A standard curve was generated using gallic acid (GA) ranging from (0 – 500  $\mu$ g/mL) with results expressed as mg/mL GA equivalents.

### **2.2.2 Total monomeric anthocyanins by pH differential method**

#### **Potassium chloride (KCl) buffer solution (0.025 M; pH 1)**

KCl (1.86 g) was dissolved in 960 mL of water. The pH of the solution was adjusted to pH 1 with concentrated HCl. The buffer solution was then transferred to a volumetric flask (1 L) and water was added to make up to 1 L. The buffer solution was stored at room temperature and the pH was checked each time before use.

#### **Sodium acetate buffer (0.4 M; pH 4.5)**

Sodium acetate (tri-hydrate)  $\text{CH}_3\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$  (54.43 g) was mixed with 960 mL of water. The pH of the buffer solution was adjusted to pH 4.5 with concentrated HCl. The buffer solution was then transferred to a volumetric flask (1 L) and water was added to make up to 1 L. The buffer was stored at room temperature and the pH was checked each time before use.

#### **Procedure**

The pH differential method is based on the structural change of the monomeric anthocyanins as a function of pH. The coloured oxonium chromophore of anthocyanins appears at pH 1.0, whereas the colourless hemiketal forms at pH 4.5. Thus, the difference in absorbance at the  $\lambda_{\text{vis-max}}$  (520 nm) of the pigment is proportional to the concentration of pigment (Lee et al., 2019).



acetonitrile/ water/formic acid (50:49.5:0.5). The gradient conditions were as follows: the initial condition started with 92 % A and was increased to 18 % solvent B at 5.32 min, 32 % B at 27.36 min, 60 % B at 42.56 min, reaching 100 % B at 48.64 min, held at 100 % B for 6.08 min, and returning to the initial conditions for 5 min for the next analysis. The chromatographic separation was performed on a Phenomenex Gemini C18 column (5  $\mu$ m, 250 mm x 4.6 mm) at a flow rate of 0.5 mL/min. The temperature of the column was maintained at 35 °C and the injection volume was 10  $\mu$ L. The compounds in the samples were identified and quantified based on the available standards, molecular mass and structure.

#### **2.2.4 Determination of sugars using HPLC-evaporative light scattering detector (ELSD)**

The principle of is ELSD to nebulize the eluent of the target compounds from the LC into droplets that are carried by an inert gas in an evaporator tube. The mobile phase is then evaporated from the droplets which are then directed towards a light beam in the detector. The particles scatter the light beam and the amount of scattered light is a measure of the concentration of the analyte (Dreux & Lafosse, 1995).

Soluble sugars (glucose, fructose, sucrose) present in fruit concentrates were analysed by chromatographic technique as described by Ifie et al. (2016). Briefly, a UFLCXR (Shimadzu) system attached to an evaporative light scattering detector (ELSD) was used to identify and quantify individual sugars. The analysis was performed under isocratic conditions and the column used was Grace Davison Prevail Carbohydrate (5  $\mu$ m, 250 nm X 4.6mm). The mobile phase was 75% acetonitrile (v/v) with a flow rate of 0.5 mL/min and injection volume of 10  $\mu$ L. Individual sugars were quantified using external standard curves of glucose, fructose and sucrose in the range of 250 - 3000  $\mu$ g/mL with fucose being added as an internal standard.

## 2.3 Biochemical assays

### 2.3.1 Determination of $\alpha$ -glucosidase inhibition

#### **Sodium phosphate buffer (100 mM; pH 7.0)**

A stock solution of 100 mM sodium phosphate buffer solution was prepared by mixing 57.7 mL of 1 M  $\text{Na}_2\text{HPO}_4$  (di-basic) and 42.3 mL of 1 M  $\text{NaH}_2\text{PO}_4$  (mono-basic) and then stock solution diluted to obtain 100 mM of sodium phosphate buffer solution of pH 7. The buffer solution was stored at 4°C. The pH was checked each time before use.

#### **Enzyme: $\alpha$ -glucosidase solution (0.5 U/mL)**

The stock solution of  $\alpha$ -glucosidase (100 U/mL) from *Saccharomyces cerevisiae* (Sigma G5003) was prepared by dissolving 5.26 mg enzyme powder in 1 mL sodium phosphate buffer (100 mM; pH 7.0), then stock solution was diluted by 1:200 to provide 0.5/mL of solution in the enzyme assay.

#### **Substrate: p-nitrophenyl glucopyranoside (2.5 mM)**

The substrate solution was prepared by dissolving 7.55 mg *p*-nitrophenyl glucopyranoside (*p*NPG; MW 301.25 g/mol) in 10 mL of sodium phosphate buffer (100 mM; pH 7.0) and used directly for analysis.

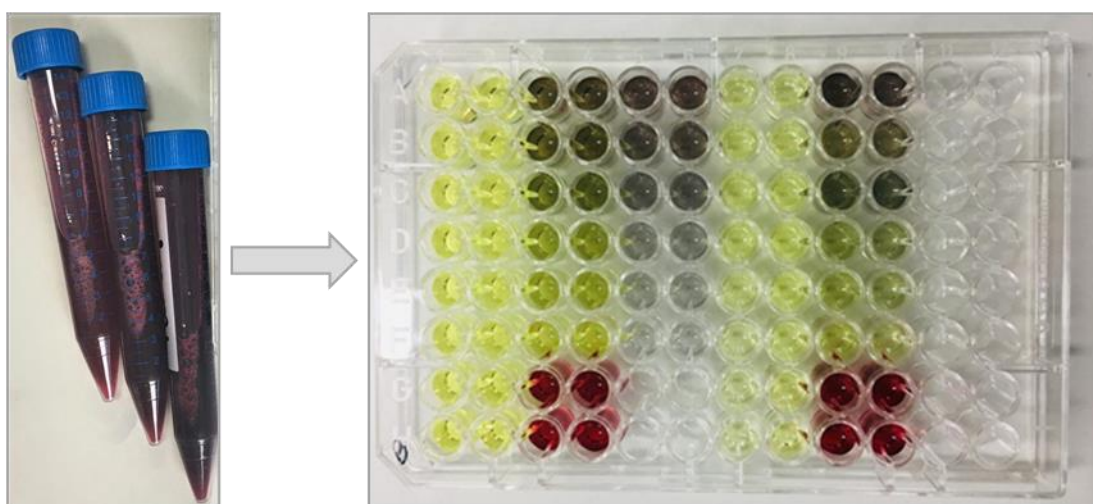
#### **$\alpha$ -glucosidase inhibition assay**

Activity of  $\alpha$ -glucosidase enzyme was measured according to Zhang et al. (2017) with some modifications. A range of sample (pure compounds or anthocyanins rich extracts) dilutions were prepared in 0.1 M phosphate buffer (pH 7.0). For the assay, in 96 well plate, 100  $\mu\text{L}$  of sample or pure compounds were incubated with 50  $\mu\text{L}$  of  $\alpha$ -glucosidase solution (0.5 U/mL) in 0.1 M phosphate buffer (pH 7.0) for 10 min at 37 °C. This was followed by addition of 50  $\mu\text{L}$  of substrate *p*-nitrophenyl glucopyranoside (2.5 mM) solution in 0.1 M phosphate buffer (pH 7.0). The change in absorbance of released *p*-nitrophenol was recorded at 405 nm in 1 min intervals over a 10 min period (at 37 °C) using Tecan Spark 10 M multimode microplate reader (TECAN, Mannedorf Switzerland). A reaction product scan (360-600 nm) was

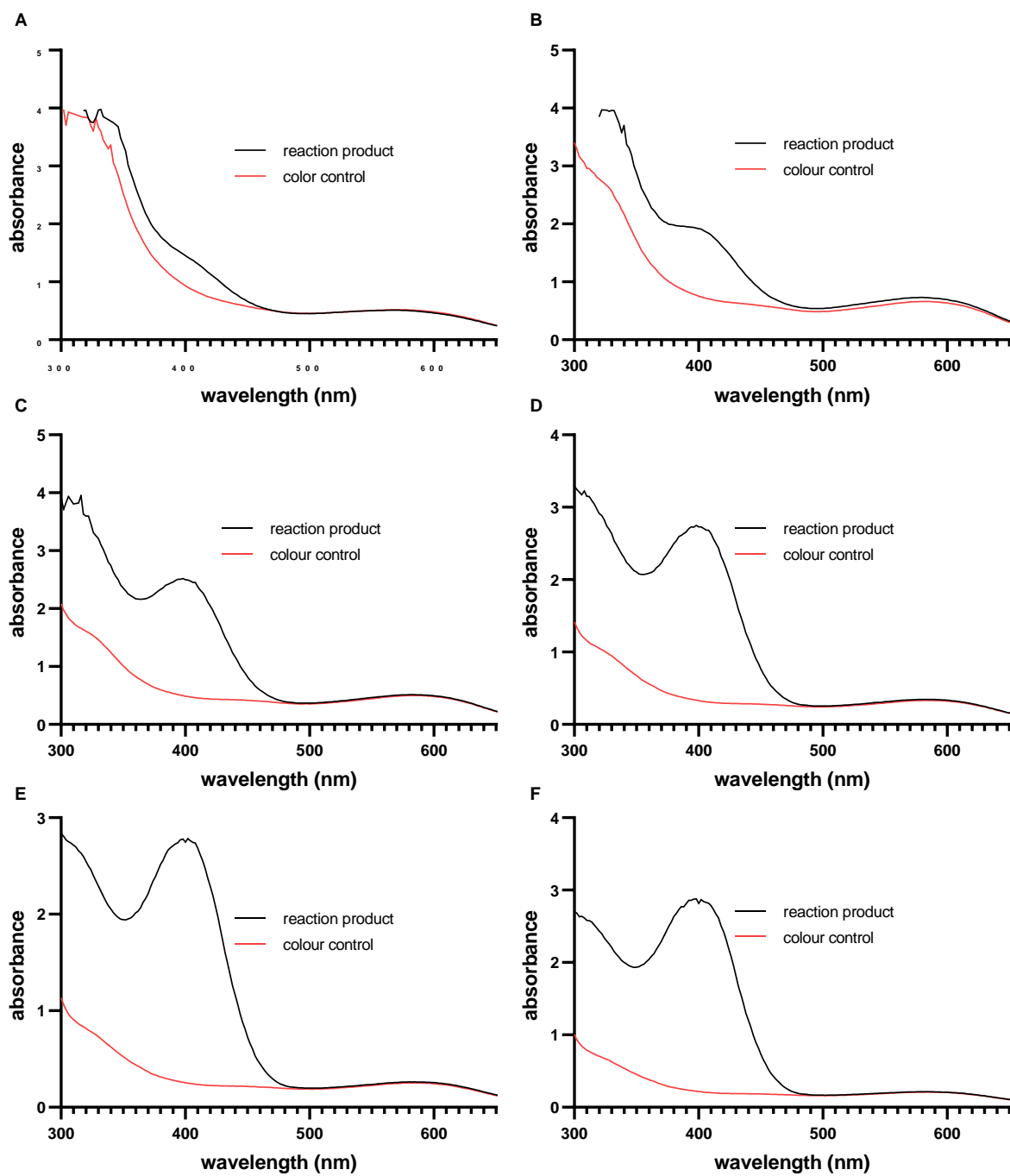
performed at the end of each experiment. The rate of enzyme inhibition in per cent was calculated from the change in absorbance in comparison to control, by subtracting the absorbance of sample from the non-inhibited control, divided by the control value. Acarbose, a synthetic inhibitor of  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes, was used as a positive control (0-4000  $\mu\text{g}/\text{mL}$ ).

### 2.3.1.1 Preliminary experiments

Anthocyanins are coloured compounds, responsible for dark red colour of hibiscus concentrate, cause background absorbance which may interfere with the product (*p*-nitrophenol) formation at 405 nm. Therefore, preliminary experiments were conducted with color controls (HS sample and buffer only) of different dilutions of HS under the same conditions as the enzymatic reaction (HS sample+substrate+enzyme) (Figure 2.1) and wavelength scans were recorded. The results are shown in (Figure 2.2) which indicate the absorbance signal generated at 405 nm is the result of product formation which is not affected by the absorbance of anthocyanins. Moreover, there was clear difference in the product formation at different concentrations of hibiscus indicated by absorbance values at 405 nm.



**Figure 2.1**  $\alpha$ -glucosidase measurement in HS samples with colour control.



**Figure 2.2** Wavelength scans of product formation and colour control samples of different dilutions (A-F high to lower) of HS under same experimental conditions.

### **2.3.2 Determination of $\alpha$ -amylase inhibition**

#### **Phosphate-buffered saline (PBS) (20 mM; pH 6.9)**

The PBS solution was prepared by dissolving 2.4 g of sodium phosphate and 0.3915 g of sodium chloride (NaCl) in 800 mL water (1L flask). The pH was adjusted to 6.9 with either 1 M NaOH or HCl and final volume 1 L was made with water. The pH was checked each time before use.

#### **Sodium potassium tartrate solution (5.3 M) (Reagent A)**

The solution was prepared by dissolving 120 g sodium potassium tartrate in previously heated 80 mL of 2 M NaOH at 50-70 °C. It was then heated directly on a heating/stir plate with constant stirring to dissolve taking the precaution not to boil the solution.

#### **3,5- Dinitrosalicylic acid (DNS) solution (96 mM) (Reagent B)**

The solution was prepared by dissolving 438 mg of DNS in a 200 mL of water and heated directly on a heating/stir plate with constant stirring to dissolve at 100 °C and taking the precaution not to boil the solution.

#### **Colour reagent Solution**

The colour reagent containing DNS for the final step of the assay was prepared by slowly adding reagent A to reagent B with continuous stirring. The solution was stored in an amber bottle at room temperature with expiry after 6 months

#### **Substrate: amylose stock solution (2.5 mg/mL)**

The stock solution of amylose from potato starch (Sigma A0512) was prepared by mixing 50 mg of amylose, 500  $\mu$ L ethanol, 5 mL Millipore Q water and 1 mL of 10% sodium hydroxide solution. The solution was heated on a hot plate for 15 minutes until amylose dissolved and then cooled to room temperature. The pH was then adjusted to 6.9 and the volume added up to 20 mL.

### **Porcine pancreatic amylase (100 U/mL)**

The enzyme stock solution 100 U/mL was prepared from stock solution (Sigma A6255) in PBS solution (20 mM; pH 6.9) and stored in freezer in aliquots -20 °C. The stock solution 100 U/mL was diluted by 1:80 to provide 1.25 U/mL of working enzyme solution for the DNS assay.

### **Substrate: 2-chloro-4 nitrophenyl $\alpha$ -D-maltotrioside (CNPG3) (2 mM)**

The substrate solution was prepared by dissolving 13.2 mg CNPG3 (MW 659.98) in 10 mL PBS solution (20 mM; pH 6.9) and used directly for analysis.

## **Inhibition of $\alpha$ -amylase**

### **2.3.2.1 DNS method**

The  $\alpha$ -amylase inhibitory effects of fruit concentrates were measured using DNS assay according to the procedure prescribed by Nyambe-Silavwe et al. (2015). Briefly, 50  $\mu$ L of sample, mixed with 50  $\mu$ L phosphate buffer saline (20 mM, pH 6.9) and 200  $\mu$ L of starch (amylose) solution (2.5 mg/mL) in 2 mL Eppendorf tube (safe lock), were incubated at 37 °C for 10 min. The reaction was started by the addition of 200  $\mu$ L porcine pancreatic  $\alpha$ -amylase (PPA) solution (1.25 U/mL) diluted in PBS and incubated at 37 °C for 10 min. Subsequently, the reaction was stopped by placing the samples in a water bath (Grant GLS Aqua 12 plus) at 100 °C for 10 min, then tubes were transferred to ice to cool down to room temperature. The samples were centrifuged using a microcentrifuge for 5 min. The samples obtained was subjected to solid phase reaction (SPE) using Oasis MAX cartridge (30 mg) in order to remove the polyphenols which may react with the DNS reagent and interfere with the assay result (Nyambe-Silavwe et al., 2015).

To the SPE purified samples, 1 mL DNS reagent was added, and the mixture was heated at 100 °C for 10 min. After cooling to room temperature, 250  $\mu$ L from each sample was placed in a 96 well plate and the absorbance was recorded at 540 nm using plate reader. The rate

of enzyme inhibition was calculated as a percentage of the control (without inhibitor) using the following equation:

$$\text{inhibition (\%)} = \left[ \text{absorbance} \frac{(\text{control} - \text{sample})}{\text{control}} \right] \times 100 \dots \dots \dots \text{(Equation 2.2)}$$

### 2.3.2.2 Direct chromogenic method

The inhibition of  $\alpha$ -amylase activity was measured according to the method described by Kalita et al. (2018) with some modifications. Briefly, in wells of a 96 well plate, 50  $\mu$ L of extract/pure compound/acarbose were incubated with 100  $\mu$ L of PPA solution (1 U/mL) in 20 mM phosphate buffer (pH 6.9) for 10 min at 37 °C. This was followed by the addition of 50  $\mu$ L of substrate CNPG3 2-chloro-4 nitrophenyl  $\alpha$ -D-maltotrioxide (2 mM, in phosphate buffer). The change in absorbance of released p-nitrophenol was recorded at 405 nm in 1 min intervals over a 10 min period (at 37 °C) using Tecan Spark 10 M multimode microplate reader (TECAN, Mannedorf, Switzerland). A reaction product scan (360-600 nm) was performed at the end of each experiment. The rate of enzyme inhibition in per cent was calculated from the change in absorbance in comparison to control, by subtracting the absorbance of sample from the non-inhibited control, divided by the control value. Acarbose, a commonly used antidiabetic drug and synthetic inhibitor of amylase, was employed as a positive control.

### 2.3.3 Enzymatic kinetics and mode of inhibition

In preliminary experiments, the optimum enzyme and substrate concentrations for the direct chromogenic assay were determined using different concentrations of  $\alpha$ -amylase (0.25, 0.5 and 1 U) and CNPG3 (0, 0.625, 1.25, 2.5 and 5 mM). The linearity of enzyme reaction was monitored over 20 min under optimized enzyme and substrate concentrations, and incubation time for enzymatic reaction was defined. In order to determine  $K_m$  and  $V_{max}$ , the selected enzyme concentration (1 U/mL) was incubated with varying concentrations of substrate (0-5 mM). The rate of enzymatic reaction ( $V$ ) was calculated by dividing the change

in absorbance (at 405 nm) over time. The Lineweaver-Burk double reciprocal plot was obtained by plotting the  $1/V$  against reciprocal of substrate ( $1/[S]$ ), and  $K_m$  and  $V_{max}$  were calculated.

To detail the inhibitory mechanism, a series of experiments were conducted by maintaining PPA at 1 U/mL and varying concentration of inhibitors (acarbose, black currant and cyanidin-3-O-galactopyranoside) and substrate CNPG3 (0-5 mM). Michaelis-Menten kinetic parameters and mode of inhibition of PPA for every inhibitor was determined from a Lineweaver-Burk.

## **2.4 *In vivo* human glycaemic study (Hibiscus and blueberry study)**

### **2.4.1 Materials**

White bread (Warburtons®), household sugar and low nitrate (<0.1 mg/L) still natural Buxton® mineral water were all purchased from a local Tesco® store (Leeds, UK). Accu-Chek® Performa Nano including test strips were purchased from a commercial provider (Boots, Leeds, UK). Microvette® 200 tubes were obtained from Sarstedt (Leeds, UK), DuoSet Insulin kit and controls were from R&D systems Bio-Techne (Abingdon, UK). Sugars (fructose, glucose) were purchased from Holland and Barrett® (Leeds, UK).

### **2.4.2 Ethical approval**

Both intervention studies were reviewed and approved by the Research Ethics Committee, University of Leeds, UK and covered under the same ethics (Ethical reference number: MEEC 16-028). The work has been carried out in accordance with the Declaration of Helsinki (The Code of Ethics of the World Medical Association).

### **2.4.3 Participant recruitment**

For each study, healthy participants (males/females), meeting the inclusion criteria (not pregnant or breast-feeding, not taking any medication or supplements) were recruited using advertisements, flyers and personal communications. The interested volunteers were tested

for their eligibility (Appendix A) given a participation information form (Appendix C). Written informed consent was obtained from each participant (Appendix D). The participants were advised to avoid the consumption of polyphenol rich foods (fruits, vegetables, tea, coffee, red wine, cocoa) and strenuous exercise prior to each visit. Subjects were asked to fast for at least ten hours and no more than twelve hours prior to each visit. Anthropometric measurements (height, weight, and waist circumference) were obtained for all subjects during their first visit. The height was measured using a stadiometer, which consists of a long ruler and headpiece. A digital scale was used to measure the weight of the participants. Using an inelastic measuring tape, the waist circumference was measured midway between the lowest ribs and the iliac crest. All participants completed a health screening questionnaire (Appendix B) prior to the first visit and a 24 h food recall questionnaire at each visit. Physical activity level was determined using IPAQ (International Physical Activity Questionnaire).

#### **2.4.4 Preparation of test drinks**

The detailed composition of test drinks/meals used in studies are explained in individual Chapters (3 & 5). Briefly, test drinks were prepared by diluting concentrated juice with low nitrate (<0.1 mg/L) still natural Buxton® mineral water. The sugar content of all the drinks were adjusted to achieve similar profile and amount of available carbohydrates. The water was used as a reference/control drink in each study.

#### **2.4.5 Blood sampling**

In the present study, blood samples were taken using fingertip capillary sampling to determine the glycaemic response. The finger-prick method is to collect blood from peripheral capillaries, and the blood glucose concentration approximates the level of arterial blood glucose (Liu et al., 1992). There are various methods for blood sampling with different sites giving different glucose concentrations. Despite of few differences in fasting glucose in capillary blood and venous blood, fingertip capillary sampling is recommended as the postprandial blood glucose concentrations in capillaries are higher than in the veins (Yang et al., 2012). Moreover, capillary blood glucose has shown to have lower coefficient of variation

(CV) and higher iAUC than venous blood (Wolever et al., 2003; Hätönen et al., 2006). While diabetes is diagnosed based on measurements of venous plasma glucose, venous blood sampling as well as finger pricks can both be used to assess glycaemic response (FAO/WHO, 1998). Blood samples should be taken at standardised times as recommended by FAO/WHO 1998, at the fasting stage (0) and then 15, 30, 45, 60, 90, 120 and 180 minutes after starting the test meal for consistent glycaemic response.

#### **2.4.6 Study protocol**

In general, each study was a randomized controlled trial in which participants attended the human study facilities at the University of Leeds, UK, on three occasions, separated by 2-3 days between each visit. On each visit, after ensuring the subjects were comfortable and warm, baseline blood glucose was measured. An alcohol swab (Universal ALCOTIP PRE-INJECTION SWABS) was used to wipe the middle or ring finger. After alcohol was dried, with the help of disposable lancet (Accu-Chek Safe-T-Pro Plus lancet, Roche diagnostics GmbH), the side of the chosen finger was pricked and first drop of blood was wiped. The next droplet was placed into test strip (Accu-Chek Aviva test strip, Roche), previously inserted into a glucometer (Accu-Check). Blood glucose concentration was measured in  $\text{mmol L}^{-1}$ . The volunteers were asked to consume white bread as well as test drink containing either 30 mL or 50 mL amount of concentrated juice (diluted with water to 300 mL volume) or a sugar matched control drink (300 mL), within a few minutes ( $> 5$  min). Further, blood glucose levels were determined at regular intervals following drink and bread consumption (15, 30, 45, 60, 90, 120, 150, 180 min) during each visit. Small volumes of blood (100  $\mu\text{L}$ ) were collected using microvettes at each time point. Samples were centrifuged at 1300 g, 4°C for 15 min and plasma aliquots were kept frozen at  $-80^{\circ}\text{C}$  until insulin analysis. Insulin was determined via immunoassay in duplicates after appropriate dilution.

In order to minimise the large variations in the glycaemic response, it is recommended that the control and test samples should be repeated at least twice (Brouns et al., 2005). However, this represents a high burden for the participants. The inclusion of healthy subjects in the

present project will improve the GI results and reduce the variation within the subjects (Brouns et al., 2005).

#### **2.4.7 Insulin measurement by ELISA**

Immunological methods determine the concentration of insulin in the serum by its binding to insulin antibody. The most commonly used method for insulin analysis is the enzyme-linked-immuno-sorbent-assay (ELISA) (Federlin, 2012).

DuoSet ELISA is based on the direct sandwich technique (solid phase two-site enzyme immunoassay). It is based on the affinity of an antibody (ABI) bound to a microplate which is specific to the antigen or molecule of interest. Plate is blocked and washed. The sample to be analysed is added into the well and there is a specific binding reaction between antibody and molecule of interest. A washing buffer step then follows and it removes any unbound molecule. A second antibody linked to an enzyme (peroxidase) is then added into the antibody-antigen complex formed in the first step. A substrate solution, 3,3',5,5'-tetramethylbenzidine (TMB), is then added and reacts with the enzyme. The reaction is stopped by adding acid and a colour develops, proportional to the amount of molecule of interest present in the well. The colour intensity is measured spectrophotometrically using a micro-plate reader at 450 nm. The molecule of interest can be quantified by using the standard curve equation obtained using the standard solutions of enclosed in the kit.

### **2.5 Statistical analysis**

All data (*in vitro* and *in vivo*) were analysed using GraphPad Prism; Version 9. and IBM SPSS statistics version 26 and are expressed as mean $\pm$ SEM. The variables were checked for normality using Histograms. A two factors repeated measure ANOVA was applied to determine the significance of intervention and intervention  $\times$  time interaction followed by *Post hoc* comparisons with Tukey's adjustments to investigate the differences between the test drinks at different time points.

Glucose concentrations at different time points were plotted against time to obtain the glucose curve. The most commonly used method to determine glucose response is that of incremental area under the curve (iAUC) which calculates only the area over the baseline (fasting blood glucose) and does not take into account the area beneath the curve. It has been reported that total AUC is a descriptive factor related to basal blood glucose level whereas incremental and positive AUC more accurately describe glycaemic response to foods (Le Floch et al., 1990). Therefore, incremental blood glucose values were calculated by subtracting baseline values from all subsequent time points and incremental area under the curve (iAUC) for each glucose curve was calculated using the trapezoid rule and data was analysed using one way ANOVA. In addition, peak plasma glucose values were identified. Values of  $P \leq 0.05$  were considered significant. *In vitro* experiments were conducted at least in thrice in duplicates. The calculated inhibition rates of enzymes at the various concentrations of blueberry and acarbose were fitted with GraphPad Prism using nonlinear curve fitting.  $IC_{50}$  (half maximal inhibitory concentration) values were then obtained from the respective curve.

## Chapter 3

### ***Hibiscus sabdariffa* inhibits $\alpha$ -glucosidase activity *in vitro* and lowers postprandial blood glucose response in humans**

#### **3.1 Abstract**

*Hibiscus sabdariffa* (HS) is a rich source of anthocyanins, associated with lowering of blood pressure and modulation of blood lipids. There is limited evidence on the effects of HS on postprandial glycaemia and/or chronic markers of glycaemic control. The current study aimed to establish *in vitro* and *in vivo* anti-diabetic properties of HS, and to investigate the contribution of individual anthocyanins to inhibit the carbohydrate digesting enzymes  $\alpha$ -amylase and  $\alpha$ -glucosidase.

A randomized controlled cross-over study was conducted to establish HS effects on postprandial glucose response. Fifteen healthy participants consumed either a low or high dose of HS drink or a sugar matched control drink, alongside a portion of white bread to provide in total 50 g available carbohydrates. Blood glucose was monitored at regular intervals over 3 h with subsequent analysis of plasma insulin. Enzyme activities were determined using absorbance based methods.

The results demonstrate significant attenuation of postprandial glucose (low and high dose) and insulin responses (high dose only) following HS consumption *in vivo* which was supported by *in vitro* dose-dependent inhibition of  $\alpha$ -glucosidase ( $IC_{50}$  120.9  $\mu$ g polyphenols/mL), but not  $\alpha$ -amylase activity. Moreover, when applied with acarbose, HS showed an increased inhibition of  $\alpha$ -glucosidase. The  $\alpha$ -glucosidase inhibitory response is likely a combined result of the different components of HS as anthocyanins individually were unable to demonstrate inhibition at concentrations below 100  $\mu$ M. In conclusion, consumption of HS demonstrates potential to beneficially impact mechanisms contributing to blood glucose regulation, and regular consumption should therefore be encouraged.

## 3.2 Introduction

T2DM is characterized by hyperglycaemia and associated with impaired insulin secretion and insulin resistance. Globally, the incidence of T2DM is increasing exponentially. Considering the significant burden on patients and public health services, there is increasing interest and demand for alternative approaches such as hypoglycaemic drugs and lifestyle modifications that may contribute to prevention and/or support controlling T2DM. In particular, reduction of postprandial glycaemia has been highlighted as an effective mechanism to maintain glucose homeostasis and reduce the risk of T2DM (Kim et al., 2016). Polyphenols, a large group of plant secondary metabolites, present in fruits and vegetables, tea and coffee, have been associated with many health benefits, amongst them prevention of diabetes, cardiovascular disease and cancer (Hanhineva et al., 2010; Coe & Ryan, 2016; Kim et al., 2016). Several mechanisms have been proposed by which polyphenols may modulate glucose metabolism, such as attenuation of carbohydrate digestion by inhibiting salivary and pancreatic  $\alpha$ -amylase enzymes, and  $\alpha$ -glucosidases in the small intestinal brush border, as well as inhibition of glucose absorption, stimulation of insulin secretion and protection of pancreatic  $\beta$ -cells against glucotoxicity (Kim et al., 2016). Several flavonoids such as naringenin, kaempferol, luteolin, apigenin, (+)-catechin/(-)-epicatechin, daidzein, and epigallocatechin gallate have been reported to inhibit starch digestive enzymes (Tadera et al., 2006). Further, Zhang et al. (2010) has identified four major active phenolic compounds, ellagic acid, cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechin present in raspberries as  $\alpha$ -glucosidase inhibitors. The influence of polyphenols on glucose transporters has been studied *in vitro* by using intestinal brush border membrane vesicles and Caco-2 cells. It has been found that quercetin and tea catechins inhibited the glucose transporters Na<sup>+</sup>-dependent SGLT1 and GLUT2 (Williamson, 2013). Anthocyanins are a polyphenol subgroup and are widely distributed in edible fruits and vegetables. Their intake has been associated with cardiovascular and metabolic health (Wallace, 2011; Bordoni et al., 2019). *In vitro* studies suggest that anthocyanins such as cyanidin and its glucosides can

inhibit the activity of amylase and glucosidase (Akkarachiyasit et al., 2010). Cyanidin-3-galactoside (IC<sub>50</sub> 0.05 mM) was found most potent intestinal glucosidase inhibitor whereas cyanidin-3-glucoside (IC<sub>50</sub> 0.30 mM) was more effective against pancreatic amylase. Modulation of enzyme activity via anthocyanins may be a relevant mechanisms to suppress postprandial glycaemia and thus reduce the risk of T2DM (Sancho & Pastore, 2012).

*Hibiscus sabdariffa* (HS), also known as Roselle or red tea, is a member of the Malvaceae family, with a unique anthocyanin profile containing delphinidin- and cyanidin-sambubiosides as main anthocyanin compounds. Intake of HS has been associated with lowering of blood pressure and blood cholesterol (Hopkins et al., 2013); indeed, a recent meta-analysis of our group has confirmed the consistent effect of HS on systolic blood pressure reduction (Ellis et al., 2021). The potential anti-diabetic effects of HS have received much less attention with so far limited research being conducted. Evidence from animal studies suggests that oral administration of HS could prevent the development of insulin resistance induced by high-fructose diets in rats (Andraini & Yolanda, 2014). In prediabetic women, the consumption of rosella tea twice a day for 14 days led to a significant decrease in fasting blood glucose (FBG) when compared to the control group (Mayasari et al., 2018). However, a recent acute study could not demonstrate significant differences of postprandial glucose and insulin (Abubakar et al., 2019). Given the limited evidence on the topic;

### **3.3 Aims and objectives**

The aim of this study was to investigate the inhibitory effects of HS and its main anthocyanins (delphinidin- and cyanidin-sambubiosides) and some of their metabolites on the activity of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes and to establish acute anti-diabetic effects on postprandial glucose *in vivo* in human volunteers.

### **3.4 Objectives**

1. To measure the blood glucose and insulin response of healthy human subjects after consuming a drink with white bread. Three drinks were tested by each participant: low and high dose hibiscus drink and a sugar match control drink.
2. To determine *in vitro* enzyme inhibition ( $\alpha$ -amylase and  $\alpha$ -glucosidase) by HS and its main anthocyanins and their metabolites.
3. To investigate the potential synergistic effects of HS with the synthetic drug acarbose.
4. To monitor the kinetics of the reaction for measuring  $\alpha$ -glucosidase activity.

### **3.5 Materials and Methods**

#### **3.5.1 Compositional analysis**

HS concentrate used in this study was kindly provided by IBIS Organics, Carlisle, UK. The total phenols, total anthocyanins and total sugars (glucose, fructose, sucrose) in HS concentrate were analysed using Folin-Ciocalteu assay, pH differential method and HPLC-ELSD method, respectively. Further details about the chemicals and procedures are available in section 2.2 Chapter 2 Materials and methods.

### **3.6 Study design**

#### **3.6.1 Control and intervention drinks**

To prepare low and high dose HS drink, 30 mL and 50 mL HS concentrate were diluted to 300 mL volume with low nitrate (<0.1 mg/L) still natural Buxton® mineral water, respectively. The control drink used in this study was water. Each drink contained 25 g household sugar and consumed with 55 g White bread (Warburtons®) to provide 50 g available carbohydrates. The composition of control and test drinks is presented in Table 3.1

**Table 3.1 Characteristics/composition of test meals/drinks**

Type of drink	fructose (g)	glucose (g)	sucrose (g)	total intrinsic sugars (g)	available CH from bread (g)	added sucrose (g)	total available CH (g)	Total PP (mg)	Total ACNs (mg)
<b>Control</b>	0	0	0	0	25	25	50	0	0
<b>Low dose</b>	0.20	0.24	0.08	0.52	25	25	50.52	230	132
<b>High dose</b>	0.34	0.40	0.13	0.87	25	25	50.87	383	220

Intrinsic sugars: sugars present in hibiscus concentrate analysed by HPLC-ELSD

PP: polyphenols

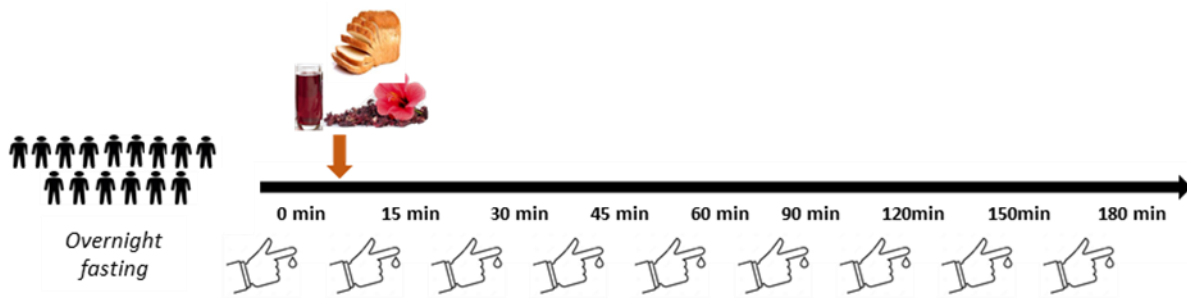
ACNs: anthocyanins

CH: carbohydrates

### 3.7 Human study: subjects and design

It was a randomized crossover trial study to assess the postprandial glycaemic response to HS in healthy adults. The intended sample size of 15 subjects was based on power calculation and similar studies to determine changes in postprandial glucose response (Torrönen et al., 2010; Torrönen et al., 2013). The study consisted of three visits; and randomisation was conducted using an online programme (<http://www.randomization.com>). Healthy participants (6 males; 9 females) aged 20-40 years with body mass index (BMI) 21-27 kgm<sup>-2</sup>, were recruited from the University of Leeds. Subjects were fasted for at least ten to twelve h before each visit. All other details are described in section 2.4 in Chapter 2 Materials and methods.

Briefly, eligible participants attended the human study facilities at the University of Leeds, UK, on three occasions, separated by 2-3 days between each visit. On the study day, participants arrived at the human study room between 8.00 and 9.00 am. Upon arriving, after a baseline blood glucose measurement, volunteers consumed 55 g of bread as well as a drink (one of HS drink or control), within a few minutes. After that, postprandial glucose was measured at 15, 30, 45, 60, 90, 120, 150, and 180 min using glucometer Accu-Chek® Performa. Small volumes of blood (100 µL) were collected using microvettes at each time point. Samples were centrifuged at 1300 g, 4°C for 15 min and plasma aliquots were kept frozen at -80°C until insulin analysis. Insulin was determined via immunoassay in duplicates after appropriate dilution.



**Figure 3.1 Human study design**

### 3.8 Statistical analysis

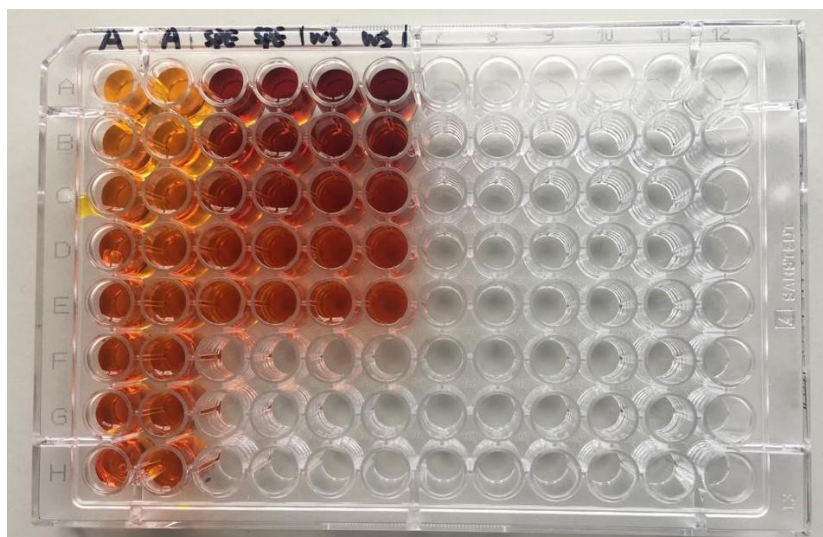
Sample size was determined to detect differences of at least one standard deviation of postprandial glycaemic response between the intervention arms. According to the calculation, the study required 13 participants for a significance level of 0.05 and a probability of 90%, with participants being controls of themselves. However, previous acute studies have shown that twelve participants on average are sufficient to detect a minimum difference of 1 mmol L<sup>-1</sup> of postprandial glucose peak response (Torrönen et al., 2010; Kerimi et al., 2017).

The significance of the overall drink x time interaction and their main effects was tested using a two factors repeated measure ANOVA and comparisons were conducted using Tukey's test, where a significant difference was observed. Postprandial blood glucose and plasma insulin incremental area under the curves (iAUCs) were calculated using the trapezoidal rule, omitting values below the baseline, over 120 and 180 minutes after consuming intervention and control drinks, and the data were analysed using one-way ANOVA. All statistical analyses were performed using SPSS (version 26, IBM), with a statistical difference of  $P < 0.05$  considered as significant. The data is expressed as mean±SEM of fifteen participants for glucose response. As a result of freezer failure during the lockdown period (Covid-19), some plasma samples were deteriorated and not analysed, therefore insulin data were expressed as mean±SEM of 9 participants.

### 3.9 *In vitro* enzyme inhibition

All the chemicals, reagents and methods used for measurement of *in vitro* carbohydrate digesting enzymes are described in detail in sections 2.3 in Chapter 2 Materials and Methods.

Acarbose, a synthetic inhibitor of  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes, was used as a positive control (0-4000  $\mu\text{g}/\text{mL}$ ). A range of concentrations of HS sample (covering a range of 0 – 400  $\mu\text{g}$  polyphenols/ $\text{mL}$ ) or pure compounds were made in 0.1 M phosphate buffer (pH 7.0) and in PBS (20 mM, pH 6.9) for  $\alpha$ -glucosidase and  $\alpha$ -amylase enzyme assay, respectively. The enzyme activities were measured and the rate of enzyme inhibition in per cent were calculated as a percentage of the control (without inhibitor).



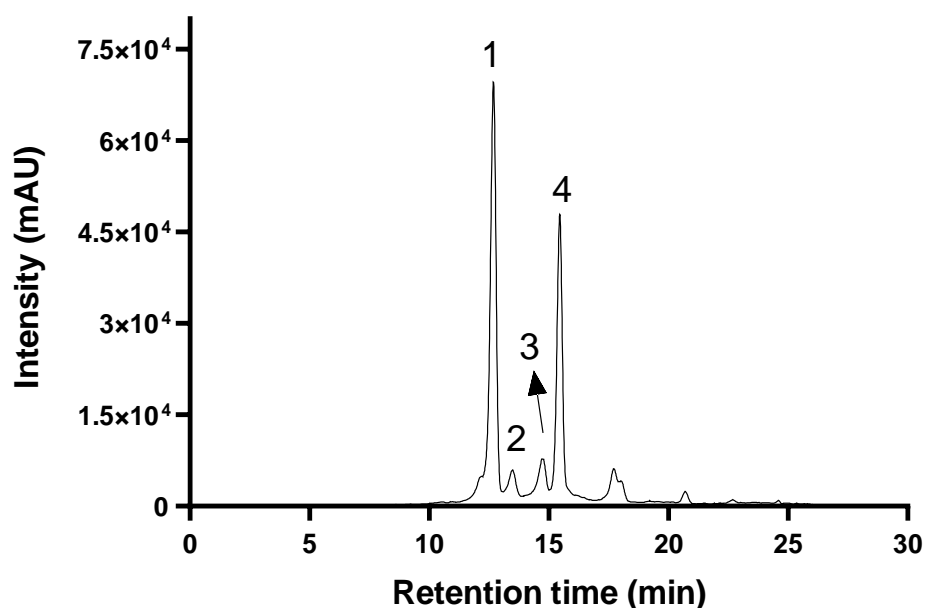
**Figure 3.2**  $\alpha$ -amylase inhibition in HS samples

## 3.10 Results

### 3.10.1 Compositional analysis of hibiscus sample

*Hibiscus sabdariffa* is a rich source of anthocyanins; HPLC/MS analysis in HS concentrate confirmed the presence of delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside as major anthocyanins, whereas delphinidin-3-O-glucoside and cyanidin-3-O-glucoside were present as minor anthocyanins (Figure 3.3). Total anthocyanin and polyphenol content are

shown in Table 3.2. In addition, sugar content was determined via HPLC-ELSD analysis which indicated the presence of low amounts of sucrose, fructose, glucose in HS concentrate (Table 3.2). However, given that the sugar content was below <1 g drinkable portion, it was not considered to have an effect on postprandial glucose response.



**Figure 3.3** Typical HPLC/MS chromatogram ( $\lambda = 520 \text{ nm}$ ) of anthocyanins distribution in *Hibiscus sabdariffa*. Peaks refer to the following compounds: 1: delphinidin sambubioside, 2: delphinidin glucoside, 3: cyanidin glucoside, 4: cyanidin sambubioside.

**Table 3.2 Compositional analysis of HS concentrate**

Ingredient	mg/mL
Total polyphenols	$7.66 \pm 0.15$
Total anthocyanins	$4.4 \pm 0.03$
Total sugar	$17.42 \pm 0.74$
Sucrose	$2.63 \pm 0.14$
Glucose	$8.00 \pm 0.40$
Fructose	$6.78 \pm 0.22$

The results are mean $\pm$ SEM of three independent measurements

### 3.10.2 Human study: glycaemic response

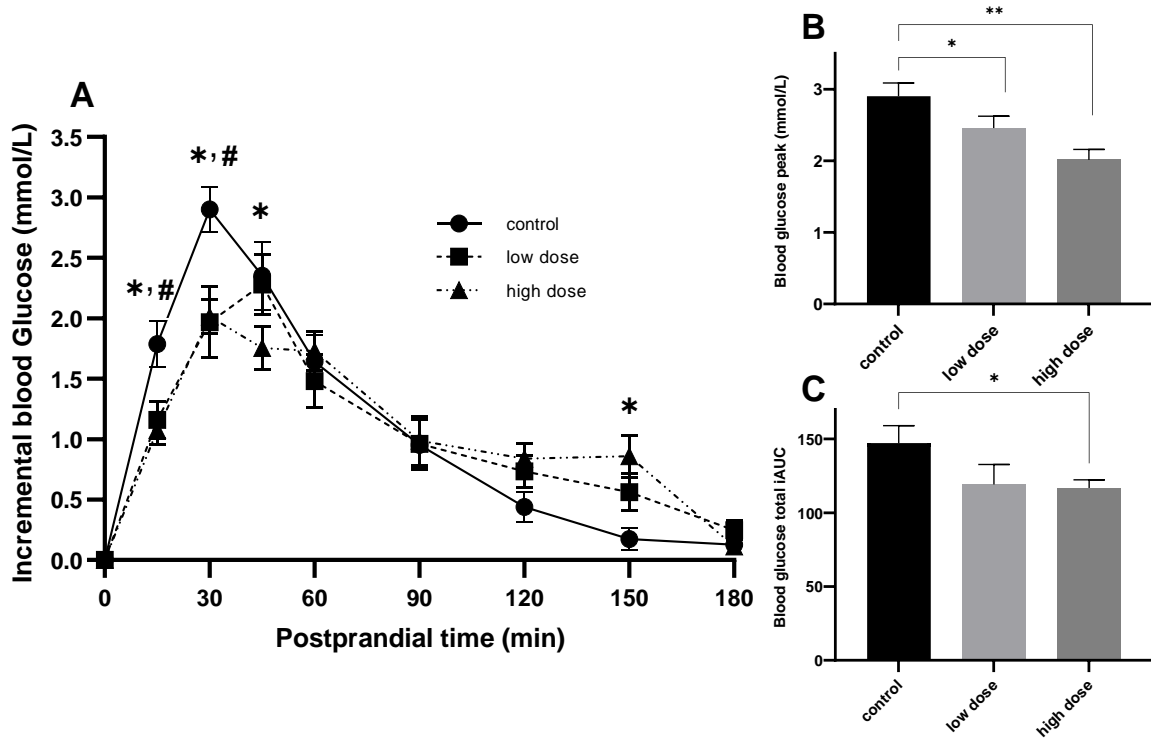
Fifteen volunteers who met the edibility criteria were randomized to test drinks and completed the study. Baseline characteristics of study participants are shown in Table 3.3

**Table 3.3 Characteristics of participants**

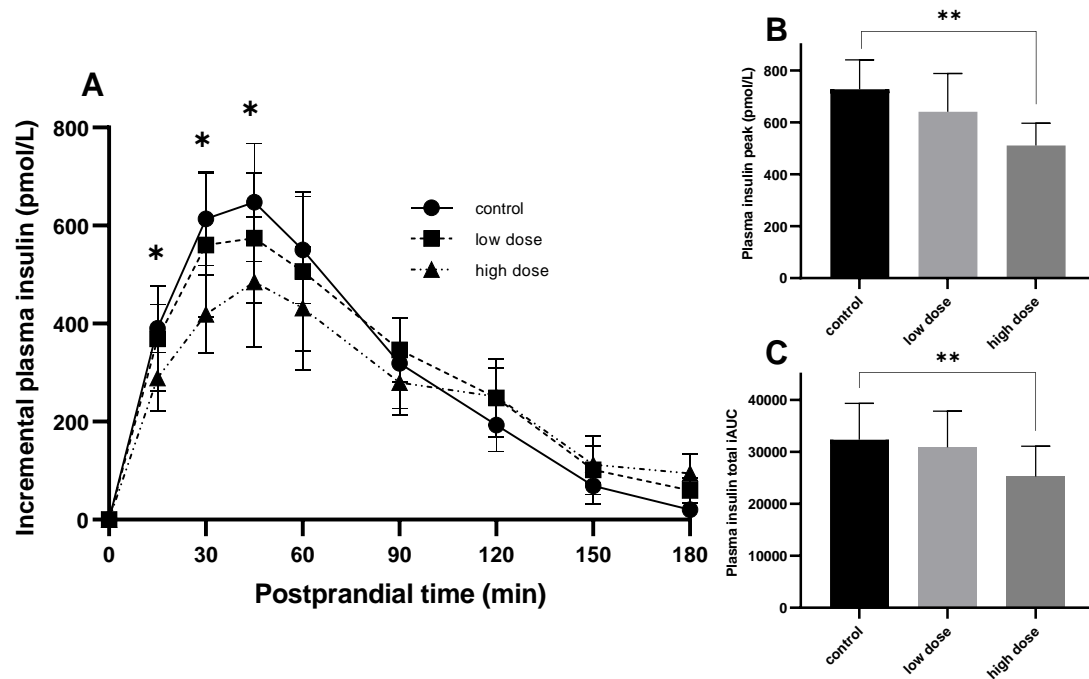
	mean±SEM
<b>Ethnicity</b>	Mixed
<b>Gender</b>	6 male/9 female
<b>Age (years)</b>	28.5±1.40
<b>Height (cm)</b>	168.5±0.08
<b>Weight (kg)</b>	69.1±2.49
<b>BMI (kg/m<sup>2</sup>)</b>	24.3± 0.61
<b>Fasting blood glucose (mmol/L)</b>	5.2±0.12
<b>Activity level</b>	4 low; 10 medium; 1 high

The postprandial changes in blood glucose after consuming control/HS drink with 50 g available carbohydrates are shown in Figure 3.4. Low and high dose treatments significantly lowered plasma glucose concentrations in the early postprandial period (0-30 min) compared with control ( $P < 0.005$ ). Post hoc analysis with Tukey's adjustment showed significantly lower glucose concentrations following high dose HS drink compared with control at 15, 30, 45 and 150 min post consumption (Figure 3.4A). The maximal plasma glucose concentration ( $C_{max}$ ) was also decreased by low (mean difference 0.93 mmol/L;  $P = 0.039$ ) and high (mean difference 0.89 mmol/L;  $P = 0.003$ ) HS dose vs control (Figure 3.4B). The total glucose iAUC (0-120 min) was dose-dependently decreased by 7 and 18% for low and high HS dose, respectively, which was significant ( $P = 0.010$ ) only for the high dose as compared to control (Figure 3.4C). Postprandial plasma insulin data, shown in Figure 3.5, indicate that high HS dose significantly lowered plasma insulin concentrations in the early postprandial period (0-45 min,  $P < 0.05$ ) whereas the low HS dose had no significant effect on plasma insulin ( $P > 0.05$ ) compared with the control (Figure 3.5A). This was also reflected in plasma peak insulin (Figure 3.5B) and total insulin iAUC (0-120 min) (Figure 3.5C) which were both significantly lower

following high HS intake ( $P = 0.048$ ,  $P = 0.010$ ) but not after low HS dose ( $P = 0.591$ ,  $P = 0.099$ ).



**Figure 3.4** Incremental blood glucose response over 180 min following consumption of control drink (●) and either low (■) or high dose hibiscus (▲) sugar matched drinks (A); as well as peak plasma glucose (B) and total iAUC (C). The data represent means with SEM of 15 participants. Post hoc analysis of time-point differences in change from baseline in glucose with Tukey's adjustment at  $P < 0.05$  was done for control compared with low and high dose of hibiscus. The symbols \* and # indicate the significant difference between control and low and high dose of hibiscus, respectively.



**Figure 3.5** Incremental plasma insulin response over 180 min following consumption of control drink (●) and either low (■) or high dose hibiscus (▲) sugar matched drinks (A). (B) indicates plasma peak insulin concentrations and (C) total iAUC. Data are mean with SEM of 9 participants. Post hoc analysis of time-point differences for change of insulin, with Tukey's adjustment at  $P < 0.05$  was done for control compared with low and high dose of hibiscus. The symbols \* indicates the significant difference between control and high dose of hibiscus. No significant difference ( $P > 0.05$ ) was observed after intake of low dose of hibiscus as compared to control.

### 3.10.3 Inhibition of $\alpha$ -glucosidase and $\alpha$ -amylase in vitro

Inhibition of  $\alpha$ -glucosidase was determined by the rate of release of *p*-nitrophenol (yellow colour compound) from *p*-nitrophenyl glucopyranoside at 37 °C. As shown in Figure 3.6A acarbose, a synthetic inhibitor of  $\alpha$ -glucosidase which was used as positive control, dose-dependently inhibited enzyme activity with  $IC_{50}$   $619 \pm 1.76$   $\mu$ g/mL. The detection wavelength of 405 nm that is used in this assay to determine product formation is a potential cause of interference when measuring inhibitory activity of natural pigments i.e., anthocyanins, which show considerable absorbance signal at this wavelength.

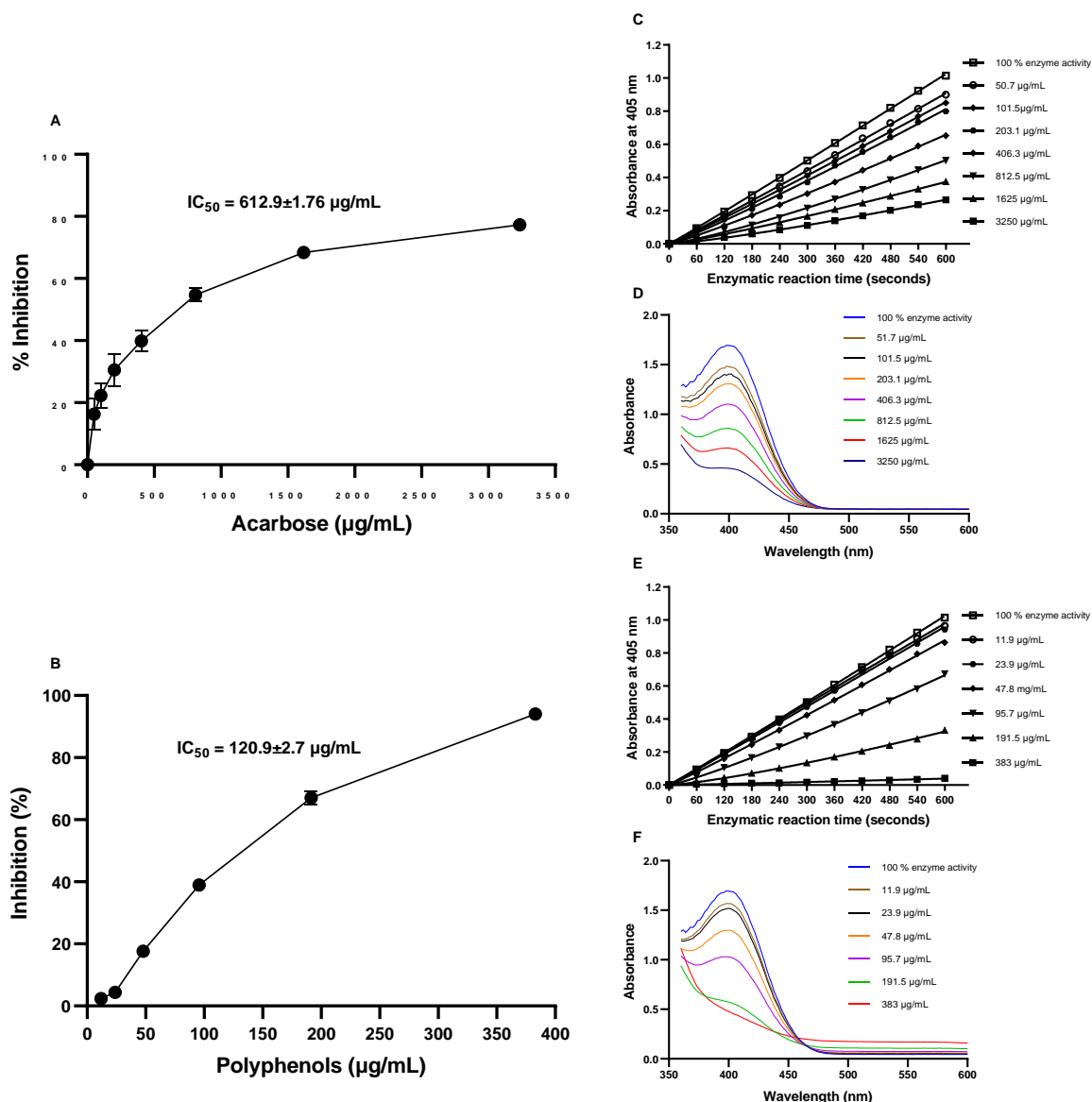
Therefore, the change in absorbance resulting from *p*-nitrophenol release, was recorded at 405 nm over a 10 min reaction period and plotted against time. For acarbose, the results showed linearity with  $r > 0.99$  for all samples (Figure 3.6C). In addition, wavelength scans (350-600 nm) confirmed the dose-dependent reduction in reaction product formation in the presence of inhibitor whereas maximum *p*-nitrophenol was released without acarbose (buffer+enzyme+substrate, 100% enzyme activity), shown in Figure 3.6D. As shown in Figure 3.6B, HS concentrate also inhibited  $\alpha$ -glucosidase activity in a dose-dependent manner with  $IC_{50}$   $120.9 \pm 2.7$   $\mu$ g polyphenols/mL. Similarly to acarbose, the absorbance readings demonstrate linearity over time (Figure 3.6E;  $r > 0.99$ ) and clearly document product formation inhibition through wavelength scan (Figure 3.6F) in these samples which were corrected for their individual background absorbance.

In order to identify the contribution of individual anthocyanins to  $\alpha$ -glucosidase inhibition, the potential of delphinidin- and cyanidin sambubiosides and their metabolites protocatechuic acid, gallic acid and chlorogenic acid, were investigated. The results indicated that the pure anthocyanins did not exert enzyme inhibition up to a concentration of 100  $\mu$ M); and only a weak inhibition (<10%) was observed for the phenolic acids at 100  $\mu$ M (Appendix F).

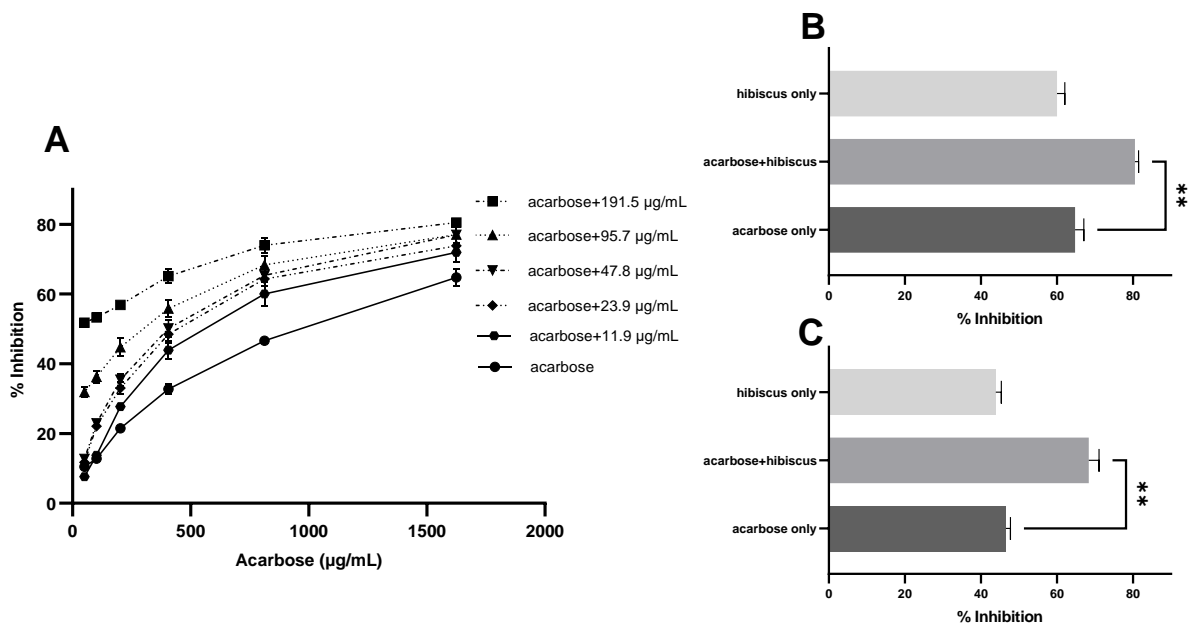
In addition, we aimed to establish whether HS and acarbose might act synergistically on  $\alpha$ -glucosidase. Therefore, the assay was performed in the presence of both, acarbose and different concentrations of HS. As results in Figure 3.7A demonstrate, the dose-dependent glucosidase inhibitory effect of acarbose was enhanced by increasing amounts of HS in the

reaction. As shown in Figure 3.7B & C, the addition of HS (191.5 and 95.7  $\mu\text{g}/\text{mL}$ ) to the assay mixture in combination with acarbose increased enzyme inhibition by 24 and 19%, respectively, as compared to acarbose alone.

Similarly, acarbose dose-dependently inhibited  $\alpha$ -amylase enzyme ( $\text{IC}_{50}$  37.6  $\mu\text{g}/\text{mL}$ ), however, neither HS concentrate (up to 400  $\mu\text{g}$  polyphenols/ $\text{mL}$ ) nor individual anthocyanins or their metabolites (up to 100  $\mu\text{M}$ ) had an effect on enzyme activity.



**Figure 3.6** Dose dependent inhibition of  $\alpha$ -glucosidase enzyme by acarbose (A) ranging from 0-4000  $\mu\text{g/mL}$  and HS extract (B) ranging from 0-400  $\mu\text{g}$  polyphenols /mL. The results are expressed as mean with SEM of three independent measurements performed in duplicate. Kinetic measurement of  $\alpha$ -glucosidase activity inhibition for acarbose (C, D) and HS extract (E, F) are presented. Data recording was performed per minute over a total period of 10 min (B, E) followed by wavelength scan (C, F) of each sample in the visible range (350-600 nm) to confirm reaction product p-nitrophenol. Shown is a representative set of data within one experiment.



**Figure 3.7** Effect of different concentrations of hibiscus polyphenols and acarbose on  $\alpha$ -glucosidase inhibition (A). The % inhibition at selected concentrations; 191.5  $\mu\text{g}$  polyphenols/mL HS extract + 1625  $\mu\text{g/mL}$  acarbose (B) and 95.7  $\mu\text{g}$  polyphenols/mL HS extract + 812.5  $\mu\text{g/mL}$  acarbose (C) is increased. Data are mean with SEM of three experiments performed in duplicate. \* indicates significant difference of hibiscus-acarbose combination versus acarbose ( $P < 0.05$ , t-test)

### 3.11 Discussion

This study aimed to investigate whether the acute consumption of HS drink in combination with carbohydrates could inhibit the rise in blood glucose concentrations in a healthy population. Two different doses (low and high) of HS providing 230 and 383 mg total polyphenols, and 132 and 220 mg anthocyanins, respectively, were administered to fifteen health volunteers in different sessions. Baseline and post meal consumption (up to 3 h) plasma glucose and insulin were measured. While addressing the mechanism of action of HS on hyperglycaemia, *in vitro* inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzyme activity was measured.

The current study demonstrates for the first time, that acute HS consumption can attenuate postprandial glycaemic response. In particular, the higher dose of HS significantly ( $P < 0.05$ ) reduced blood glucose (32% decrease in AUC) and plasma insulin concentrations (7% decrease in AUC) in the first 45 min of the postprandial period. The lower dose also markedly attenuated blood glucose (27% decrease in AUC) in the initial period (0-30 min) but had no effect on insulin. As a consequence of the reduced concentrations in the early phase, slightly elevated concentrations for glucose and insulin were observed in the later period (60-180 min) highlighting a delayed glucose appearance following HS consumption. These findings are consistent with previous human studies that demonstrated an extension of postprandial glycaemic response following mixed berry (Torrönen et al., 2010) and blackcurrant (Castro-Acosta et al., 2016) interventions. Anthocyanins have been associated with inhibition of the intestinal  $\alpha$ -amylase and  $\alpha$ -glucosidase activity (Adisakwattana et al., 2004; Berger et al., 2020). Furthermore, anthocyanins and other berry polyphenols have also observed to delay the glucose transport from the intestine to the blood through the inhibition of sodium glucose co-transporter SGLT1 and the glucose transporter GLUT2 (Hanhineva et al., 2010). Therefore, reduced postprandial glycemia observed in the current study may be explained by the inhibition of the  $\alpha$ -glucosidase by HS polyphenols. When analyzing the profiles of glucose and insulin (iAUC) over a 2 h postprandial period, which is a common reference point in most

postprandial trials, the consumption of HS drink results in a marked decrease of 18 and 21%, respectively, when compared to the control group.

Polyphenol-rich foods in combination with carbohydrates can reduce peak and early phase glucose response (Coe & Ryan, 2016). The degree to which this combination impacts glucose and insulin response depends on several factors such as the amount, type and source of polyphenols, the carbohydrate source, the mechanisms of action and pH of the intervention. For example, in a preliminary trial where HS tea containing 120 mg total polyphenols and 90 mg anthocyanins per portion were given alongside white bread, no changes in blood glucose were observed (Zulfiqar et al., 2019). In contrast, the present trial provided 2 and 3 times the amount of polyphenols and demonstrated a significant effect on glycaemic response with the high HS dose. These data are in agreement with previous reports where consumption of polyphenol rich berry meals or beverages, containing approximately 300 mg anthocyanins, markedly reduced the plasma postprandial glucose response, in particular in the early postprandial phase in humans (Torrönen et al., 2010; Edirisinghe et al., 2011; Torrönen et al., 2013). In a further study, blackcurrant extract providing 300 mg anthocyanins significantly reduced and delayed the appearance of glucose in the blood, and inhibited the secretion of insulin in humans (Castro-Acosta et al., 2016).

The glycemic response might depend on anthocyanin composition and presence of other polyphenols in intervention foods/beverages. Blackcurrants in comparison to lingonberries, have been found more effective to lower postprandial hyperglycaemia (Torrönen et al., 2012). Cyanidin-3-rutinoside, which is mainly found in blackcurrants (Nielsen et al., 2003), but not in lingonberries (Mane et al., 2011), has indeed demonstrated strong *in vitro* glucosidase inhibition and *in vivo* attenuation of sucrose-induced hyperglycemia in rats (Adisakwattana et al., 2011). Thus, specific anthocyanin profiles as well as the presence of other polyphenols are contributing differently towards postprandial glucose response.

The choice of reference food/drink as a carbohydrate source impacts on the glycaemic profile. Intake of berries alongside sucrose attenuated plasma glucose and insulin (Torrönen et al.,

2010), but berries had no effect on glucose response when consumed with starch-rich pancakes (Clegg et al., 2011). Similarly, Torronen et al. (2013) observed no difference in blood glucose response when berries were consumed with white bread as compared to the control, although the insulin response was reduced. Recently, it was shown that extracts from anthocyanin-rich red fruits such as chokeberry, pomegranate and red grapes were generally stronger inhibitors of  $\alpha$ -glucosidase than  $\alpha$ -amylase (Berger et al., 2020). Cleavage of disaccharides such as sucrose are facilitated by intestinal glucosidases. Therefore, the inhibition of  $\alpha$ -glucosidase (sucrase activity) by berries in the case of sucrose and poor inhibition of  $\alpha$ -amylase in the case of starch-rich bread could be the possible explanations for this effect. Therefore, in the current trial, in line with *in vitro* experiments, the carbohydrate source for the *in vivo* study included a large proportion of disaccharides (sucrose) to determine a correlation between the *in vitro* and *in vivo* methods of measuring glucose response. Indeed, the *in vitro* results from the current trial are supporting the *in vivo* findings.

Currently used antihyperglycemic drugs such as acarbose, miglitol and voglibose reduce the progression of diabetes primarily by interfering with the carbohydrate-digesting enzymes thereby leading to reduced glucose appearance in the blood (Raptis & Dimitriadis, 2001; Sudhir & Mohan, 2002; Control & Prevention, 2013). Importantly, daily intake of acarbose for 3 years reduced the risk for developing T2DM diabetes by 6% compared to control (Nijpels et al., 2008). However, these commercially available synthetic inhibitors have side effects (such as nausea, abdominal pain, flatulence), which has fueled the interest to investigate the potential of natural sources as possible alternatives. In recent years, polyphenols have been highlighted as potential  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors, as an alternative to acarbose (Tadera et al., 2006). In particular, anthocyanin-rich berries such as raspberries, strawberries, blueberries and black currant have demonstrated inhibitory properties towards  $\alpha$ -amylase and  $\alpha$ -glucosidase *in vitro* and *in vivo* (McDougall et al., 2005; Castro-Acosta et al., 2017). The ability of berries to inhibit  $\alpha$ -glucosidase was related to their anthocyanin content as described by (McDougall et al., 2005). Anthocyanins such as cyanidin-3-rutinoside and cyanidin-3-

galactoside (IC<sub>50</sub> 0.05 mM against intestinal sucrase) have been reported as *in vitro*  $\alpha$ -glucosidase inhibitors (Adisakwattana et al., 2004; Adisakwattana et al., 2009). Cyanidin-3-rutinoside is one of the major anthocyanins in blackcurrants, and it showed  $\alpha$ -glucosidase inhibitory activity (IC<sub>50</sub> 19.7  $\mu$ M) comparable with voglibose (IC<sub>50</sub> 23.4  $\mu$ M) (Adisakwattana et al., 2004).

The present study investigated the effect of HS and its anthocyanins for intestinal  $\alpha$ -glucosidase and pancreatic  $\alpha$ -amylase inhibitory activities. Current *in vitro* data show that HS concentrate is a potent  $\alpha$ -glucosidase inhibitor (IC<sub>50</sub> 120.9  $\mu$ g polyphenols/mL), lower than the synthetic inhibitor acarbose (619  $\mu$ g/mL). The findings are in line with the literature indicating the dose dependent inhibition of  $\alpha$ -glucosidase by different varieties of HS. The IC<sub>50</sub> values of the dark and light red varieties were 165 and 133  $\mu$ g polyphenols/mL, respectively (Ifie et al., 2016). Enzyme inhibitory properties of cold and hot aqueous preparations of HS, were reported with IC<sub>50</sub> of 627  $\mu$ g/mL and 723  $\mu$ g/mL, respectively (Rasheed et al., 2018). In contrast,  $\alpha$ -amylase, determined via DNS assay using porcine pancreatic and human salivary enzyme, did not show any inhibitory properties through HS or any of the anthocyanins and metabolites tested (up to 100  $\mu$ M). Similarly, negligible inhibition (<10%) of human salivary  $\alpha$ -amylase by HS extract was previously reported (Ifie et al., 2016). The results of the present trial indicate that pure anthocyanins and their metabolites exert very weak (<10%) inhibition of  $\alpha$ -glucosidase up to a concentration of 100  $\mu$ M.

This result apparently contradicts earlier published data on anthocyanins; showing  $\alpha$ -glucosidase inhibition by cyanidin sambubioside (IC<sub>50</sub> 543  $\mu$ M) and delphinidin sambubioside (756  $\mu$ M), albeit high IC<sub>50</sub> values. Different assay conditions such as substrate (maltose vs synthetic) as well as enzyme (rat intestinal glucosidase vs yeast derived) applied in previous experiments could be possible explanations for this. The combined inhibitory effects of HS and acarbose against  $\alpha$ -glucosidase demonstrated a significant decrease in enzyme activity particularly at higher concentrations of HS. Previously, synergistic effects of berry-derived anthocyanins such as cyanidin-3-galactoside with acarbose has been reported

(Adisakwattana et al., 2009). Cyanidin-based anthocyanins have been determined for their combined effect with acarbose against  $\alpha$ -glucosidase and  $\alpha$ -amylase (Adisakwattana et al., 2009; Akkarachiyasit et al., 2010; Akkarachiyasit et al., 2011).

Besides polyphenols/anthocyanins, other properties of foods/beverages, such as pH, have been reported as contributors to lower activity of carbohydrate digesting enzymes. Freitas and Le Feunteun (2018) has recently demonstrated that combining starchy food with an acidic drink (lemon juice ; pH < 3.5) *in vitro* reduced the salivary amylase activity in the stomach due to premature acidification of gastric content thereby leading to attenuated starch hydrolysis. In line with these findings are the results of an *in vivo* trial (Freitas et al., 2021) where only lemon juice in contrast to water or tea (alongside bread consumption), showed a reduced (and delayed) peak blood glucose concentration (Freitas et al., 2021). These findings indicate that lowering the pH of a starch-rich meal appears to be an effective way to attenuate the glycaemic response. The pH (2.6 – 2.7) of HS drinks could impact the salivary  $\alpha$ -amylase activity indirectly as there was no direct effect on pancreatic  $\alpha$ -amylase activity *in vitro*. However, *in vitro*  $\alpha$ -glucosidase inhibitory properties of HS, as evidenced in the current study, were confirmed by others under pH-buffered conditions in the reaction mixture (6.5-7.0). In summary, in the current study, it is likely that both the low pH of the HS intervention in combination with a pH-independent inhibition of hibiscus, on salivary  $\alpha$ -amylase and intestinal  $\alpha$ -glucosidase, respectively, has contributed to lower the postprandial glucose response *in vivo*. Further studies are needed to better understand the contribution/relevance of pH in contrast and/or in combination with bioactive effects in the regulation of glucose metabolism. The lower concentrations of individual anthocyanins (<100  $\mu$ M) tested for *in vitro*  $\alpha$ -glucosidase inhibition compared to those present in HS drink (>100  $\mu$ M) used for the *in vivo* study could explain the lack of inhibitory activity of these compounds. In addition, the combined effect of different types of polyphenols present in HS against digestive enzymes has not been considered in this study which could be another reason for the lack of inhibitory effect of HS anthocyanins on digestive enzymes.

Our data clearly support proposed anti-diabetic properties of HS when investigated in an acute setting. Importantly, long-term effects on glucose metabolism will need to be established. Our recent systematic review and meta-analysis of chronic HS intervention trials has found no differences in fasting blood glucose among the small number of studies on this topic, however the direct comparison was not possible due to variations in study design and in control groups (Ellis et al., 2021). Therefore, further studies investigating chronic effects of HS intake on markers of glycaemia are urgently needed.

### **3.12 Conclusion**

Consumption of *Hibiscus sabdariffa*, a rich source of anthocyanins and other bioactive compounds, has markedly attenuated the post meal elevation of blood glucose and insulin, a finding that can at least partially be explained by the inhibition of  $\alpha$ -glucosidase enzyme activity, although additional factors such as low pH of the HS intervention, are likely contributors to this outcome. In addition, the enhanced effect of acarbose and HS combination towards  $\alpha$ -glucosidase inhibition emphasizes the potential of HS to support conventional treatment approaches for the prevention and/or management of diabetes. Further research is warranted to better understand the mechanisms by which HS components and its metabolites contribute to beneficially modulate glucose metabolism in the short and long term.

## Chapter 4

# Comparison of a direct chromogenic assay with 3,5-dinitrosalicylic acid (DNS) assay to determine $\alpha$ -amylase inhibitory properties in pigmented samples

### 4.1 Abstract

Inhibition of carbohydrate digestion is considered as indicator for anti-diabetic properties of bioactive compounds which is gaining much interest as a potential strategy to counteract T2DM. The most relevant enzyme is  $\alpha$ -amylase which initiates starch breakdown. Indeed,  $\alpha$ -amylase inhibition has been demonstrated by different polyphenols, however screening of large sample numbers is time-consuming with commonly used assays such as the 3,5-dinitrosalicylic acid (DNS) assay and might suffer from interferences, in particular with coloured samples. The present study therefore aimed to compare the performance of a direct chromogenic assay, using 2-chloro-4 nitrophenyl  $\alpha$ -D-maltotriose (CNP3) as a substrate, with the DNS assay. The direct chromogenic assay demonstrated higher sensitivity to determine  $\alpha$ -amylase inhibition in a range of samples, including acarbose, pure anthocyanins and anthocyanin-rich samples. The direct chromogenic assay has shown to be easy to perform, fast, and reproducible, and could therefore be recommended, in particular for high-throughput applications.

### 4.2 Introduction

Anthocyanins, a sub-class of flavonoids, are water soluble naturally occurring bioactive compounds responsible for the red to dark blue colour of most flowers, fruits, and vegetables (Khoo et al., 2017). Over the last few decades, anthocyanins have been extensively studied for their diverse beneficial health effects such as antioxidant, antibacterial, antiviral, anti-inflammatory, and antidiabetic activity (Konczak & Zhang, 2004). Epidemiological studies have suggested that the consumption of foods rich in anthocyanins lowers the risk of T2DM and its complications (Putta et al., 2018). In conjunction with insulin resistance, chronic hyperglycaemia can result in pancreatic  $\beta$ -cell dysfunction or impaired insulin secretion, which

cause T2DM. Intake of diets/foods rich in bioaccessible starches and sugars are positively associated with elevated postprandial blood glucose concentrations (Aguiar & Cazarin, 2021). The inhibition of carbohydrate digestive enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase is one of the proposed mechanisms to control postprandial hyperglycaemia and the mechanistic basis for antidiabetic properties of many plants and their products (Akkarachiyasit et al., 2010).

The digestion and absorption of carbohydrates involves the hydrolysis of complex polysaccharides into absorbable monosaccharides. Dietary starch is hydrolysed by salivary and pancreatic  $\alpha$ -amylases in the mouth and small intestine, respectively, to its respective smaller fragments such as maltose and dextrin. The resultant product of  $\alpha$ -amylase action is further hydrolysed by intestinal  $\alpha$ -glucosidases into absorbable glucose units (Williamson, 2013). Although different amyolytic enzymes participate in the process of starch breakdown, the contribution of  $\alpha$ -amylase is the most important for the initiation of this process (Freitas & Le Feunteun, 2019). Therefore, inhibition of starch hydrolysis through  $\alpha$ -amylase inhibition seems to be an effective way to control and prevent the risk factors associated with diabetes mellitus. Antidiabetic drugs, such as acarbose, miglitol and voglibose have been approved to use for managing diabetes. They are enzyme inhibitors and reduce postprandial hyperglycaemia by modulating the activity of digestive enzymes (Ueno et al., 2015; Sagandira et al., 2021). However, these synthetic inhibitors demonstrate side effects such as nausea, abdominal pain, flatulence, which derives the need to investigate natural sources as possible alternatives. In recent years, polyphenols and in particular anthocyanins have been heavily investigated as potential  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors for the management and risk reduction of T2DM diabetes, as alternatives to pharmaceutical treatments such as acarbose (Adisakwattana et al., 2004; Adisakwattana et al., 2009; Da Silva Pinto et al., 2010; Zhang et al., 2010).

There are several methods reported in the literature to determine  $\alpha$ -amylase inhibitory properties of polyphenolic compounds, such as the 3,5-dinitrosalicylic acid (DNS) assay, the Nelson-Somogyi reagent, iodine-starch test, turbidity test, and chromogenic methods

(Visvanathan et al., 2020). These assays use different substrates, including starch, amylose, amylopectin, and some chemically modified derivatives of polymers and malto-oligosaccharides of varying chain length linked to chromophores, such as 4-nitrophenyl or 2-chloro-4-nitrophenyl (Tadera et al., 2006; Nyambe-Silavwe et al., 2015; Khadayat et al., 2020; Visvanathan et al., 2020; Visvanathan et al., 2021). The most commonly used assay for measuring  $\alpha$ -amylase inhibition involves the DNS reagent for detection of reducing sugars. The carbonyl end of the reducing sugars participates in a oxidation-reduction reaction with the aromatic DNS reagent to yield the deep-orange-coloured 3-amino-5-nitrosalicylic acid (ANS), which absorbs light strongly at 540 nm (Miller, 1959). However, the reducing potential of dietary polyphenols enables them to take part in the oxidation-reduction reaction, interfering with the colour development and thereby potentially impacting on the assay results. Solid-phase extraction has been recommended to minimize colour interference caused by polyphenols (Nyambe-Silavwe et al., 2015). The molecular size of the substrates (starch, amylose, amylopectin) is another factor affecting the outcome of the DNS assay which results in under- or overestimation of reducing power (Visvanathan et al., 2020). Additionally, the structural complexity among many of the natural starches makes them less suitable for detailed kinetic and inhibition studies. In order to address these problems, and to facilitate detection of enzymatic activity, many smaller, defined substrates have been developed (Damager et al., 2004).

There is a rationale to explore the use of alternative assays using synthetic substrates in order to determine inhibition of  $\alpha$ -amylase activity by pigmented polyphenols i.e., anthocyanins. Recently, Visvanathan et al. (2021) highlighted the importance of a High-Performance Anion-Exchange chromatography coupled with Pulsed Amperometric Detector (HPAE-PAD) technique using a small chain substrate (maltoheptaoside) as a more precise and accurate method for accessing the  $\alpha$ -amylase inhibitory potential of polyphenols with minimum interference. However, preparation of different standards, the use of expensive instruments and running the tests are limitations of this method (Visvanathan et al., 2021). Some studies

have reported the use of short-chain synthetic substrates such as 2-chloro-4 nitrophenyl  $\alpha$ -D-maltotrioside (CNPG3) to measure  $\alpha$ -amylase inhibitory properties of bioactive compounds but comparison among some of these methods is lacking (Berger et al., 2020; Khadayat et al., 2020; Visvanathan et al., 2020).

### **4.3 Aims**

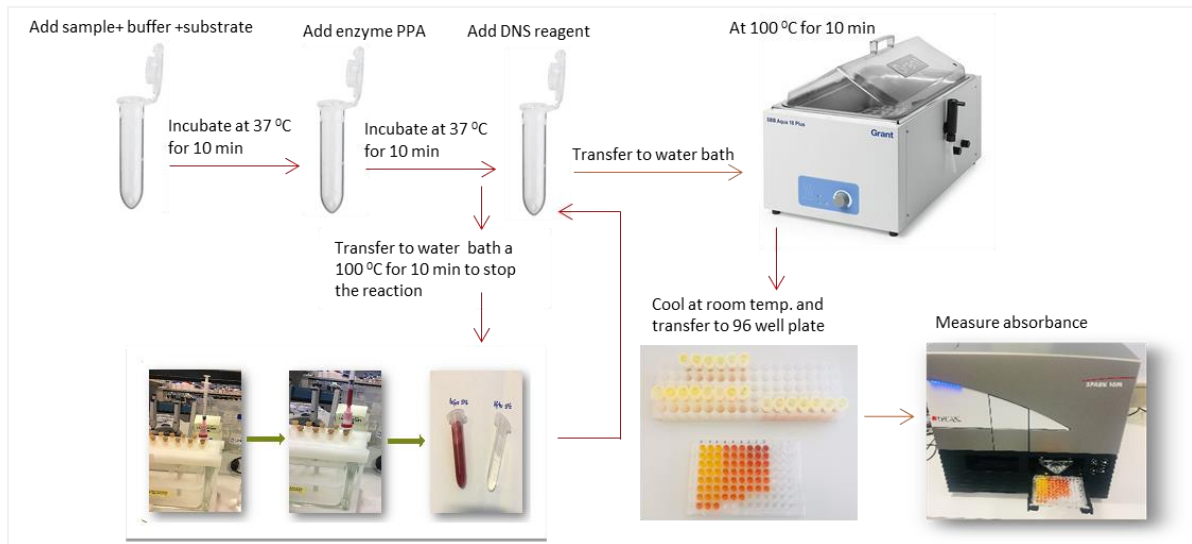
The aim of the current study was to test performance of a direct chromogenic assay that utilizes CNPG3 substrate, in comparison with the DNS assay to determine the  $\alpha$ -amylase inhibitory properties of anthocyanin-rich extracts and purified anthocyanins.

### **4.4 Objectives**

1. To establish a direct assay for measuring  $\alpha$ -amylase activity using 2-chloro-4 nitrophenyl  $\alpha$ -D-maltotrioside (CNPG3) as a substrate and porcine pancreatic amylase (PPA)
2. To determine  $\alpha$ -amylase activity by DNS method using amylose as substrate and porcine pancreatic amylase (PPA)
3. To measure and compare the enzyme inhibition by two methods and calculate  $IC_{50}$  for synthetic inhibitor (Acarbose)
4. To measure the enzyme inhibition by anthocyanin-rich extracts and pure anthocyanins
5. To determine the mode of inhibition by anthocyanin-rich extracts and pure anthocyanins

## 4.5 Materials and methods

All the chemicals and reagents used are described in detail in sections 2.2 and 2.3 in Chapter 2 Materials and methods.



**Figure 4.1** DNS assay for measuring  $\alpha$ -amylase inhibition



**Figure 4.2** Direct chromogenic assay for measuring  $\alpha$ -amylase inhibition

## 4.6 Results

### 4.6.1 Total polyphenol, total anthocyanin and total sugar contents of fruit extracts

The total polyphenol and anthocyanin contents present in different fruit extracts are shown in Table 4.1. There was significant difference ( $P < 0.05$ ) in the total polyphenols and total anthocyanins among different type of extracts apart from hibiscus and cherry which are not significantly different. The pomegranate extract has the highest amount of total polyphenols whereas as blueberry has the highest amount of total anthocyanins. The sugars (glucose, fructose, sucrose) present in fruit extracts are shown in Table 4.2. Blueberry, cherry and pomegranate have monosaccharides whereas disaccharide was only detected in hibiscus. Overall, the sugar contents of all the extracts are  $\geq 500$  mg/mL except hibiscus (Table 4.2).

**Table 4.1 Total polyphenols and total anthocyanins in fruit extracts**

Type of extract	Polyphenols (mg/mL)	Anthocyanins (mg/mL)
Blueberry	18.96 $\pm$ 0.37 <sup>b</sup>	11 $\pm$ 0.08 <sup>a</sup>
Cherry	9.83 $\pm$ 0.36 <sup>c</sup>	3.01 $\pm$ 0.02 <sup>b</sup>
Hibiscus	7.66 $\pm$ 0.15 <sup>c</sup>	4.4 $\pm$ 0.03 <sup>b</sup>
Pomegranate	23.32 $\pm$ 1.55 <sup>a</sup>	1.85 $\pm$ 0.01 <sup>c</sup>

Values are expressed as mean  $\pm$  SEM. Values in the same column followed by different superscript letters are significantly different from each other ( $P < 0.05$ ).

**Table 4.2 Sugar analysis of fruit extracts by HPLC-ELSD**

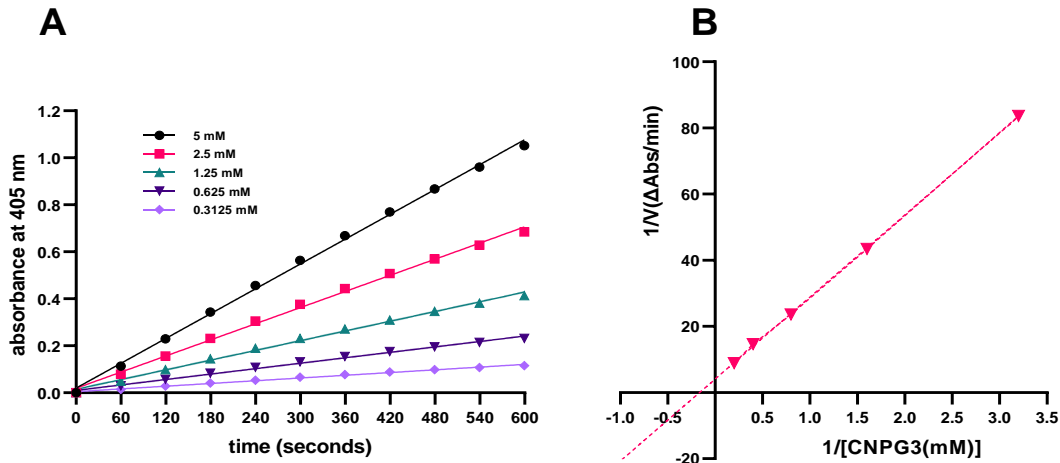
Type of extract	Fructose (mg/mL)	Glucose (mg/mL)	Sucrose (mg/mL)	Total sugars (mg/mL)
Blueberry	369.91 $\pm$ 0.40	206.69 $\pm$ 0.82	ND	576.6 $\pm$ 1.7
Cherry	229.33 $\pm$ 5.6	272.98 $\pm$ 4.5	ND	502.3 $\pm$ 0.85
Hibiscus	6.78 $\pm$ 0.13	8.00 $\pm$ 0.23	2.63 $\pm$ 0.10	17.42 $\pm$ 0.74
Pomegranate	323.17 $\pm$ 1.84	313.3 $\pm$ 4.4	ND	636.5 $\pm$ 8.82

Values are expressed as mean  $\pm$  SEM. ND; not detected.

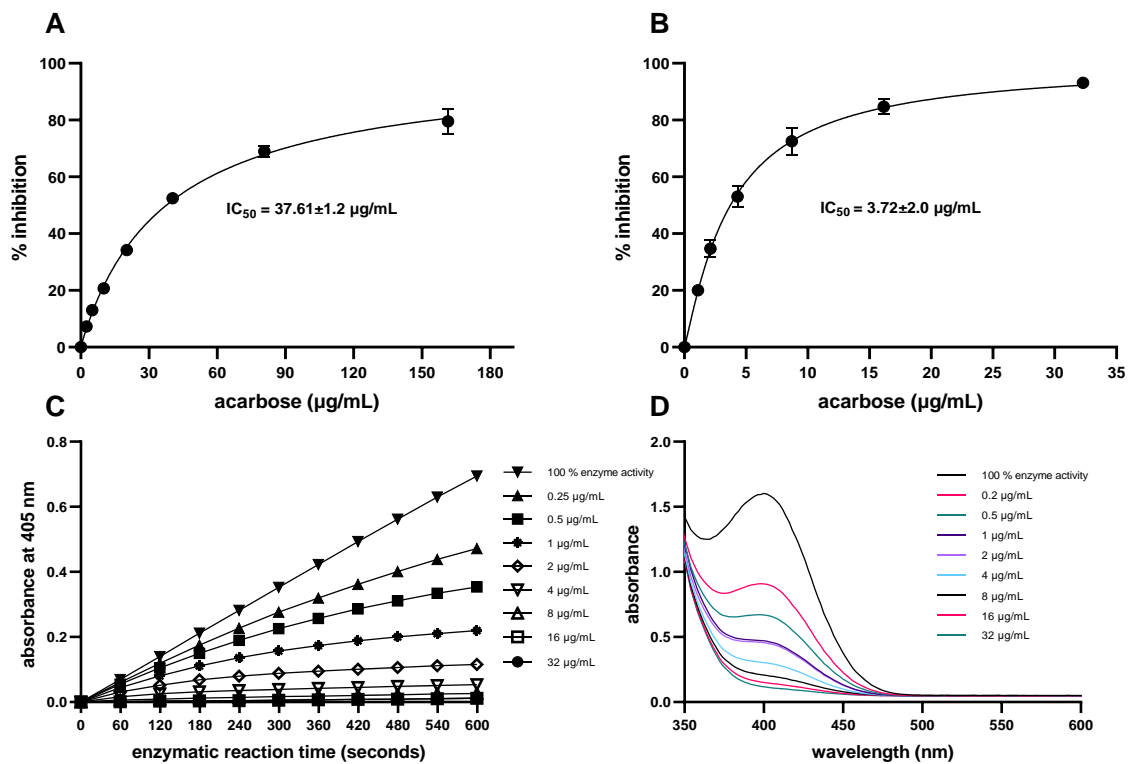
#### 4.6.2 Comparison of DNS and direct chromogenic assay for $\alpha$ -amylase inhibition by acarbose

Acarbose, a well-known  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitor, is a commonly used anti-diabetic drug (Glucobay, Precose), and being typically used as a positive control for assessing enzyme inhibitory properties of plant extracts *in vitro* (Akkarachiyasit et al., 2011; Kalita et al., 2018). Indeed, in the present study, acarbose was used as a reference in both assays, allowing comparison of  $\alpha$ -amylase inhibition using DNS and the direct chromogenic method. While acarbose inhibited  $\alpha$ -amylase activity in both assays, results from the DNS assay (covering a concentration range from 1-100  $\mu$ M), demonstrated 50% enzyme inhibition ( $IC_{50}$ ) at 37.6  $\mu$ g/mL (Figure 4.4A). In contrast to DNS data, the results from the direct chromogenic assay showed a much lower acarbose  $IC_{50}$  of 3.72  $\mu$ g/mL (Figure 4.4B) indicating that the direct chromogenic assay is superior to demonstrate inhibitory properties. Based on preliminary experiments, the direct chromogenic was performed under selected conditions, with 1 U/mL enzyme, 2 mM substrate and 10 min reaction time (Figure 4.3) Using Lineweaver–Burk plots (Figure 4.3B), values for  $K_m = 6.68$  mM and  $V_{max} = 0.26$  mM/min were determined.

The background absorbance of some plant bioactive compounds, i.e. anthocyanins, can cause interference at the wavelength that is used in the direct chromogenic assay (405 nm) to determine reaction product formation. Therefore, the increase in absorbance from *p*-nitrophenol release, was recorded in 1-min intervals over the 10-min reaction period and plotted against time. For acarbose (Figure 4.4C), the results showed linearity with  $r > 0.99$  for all samples. In addition, wavelength scans (350-600 nm) confirmed the dose-dependent reduction in reaction product formation in the presence of inhibitor whereas maximum *p*-nitrophenol was released without acarbose (buffer+enzyme+substrate, 100% enzyme activity), as shown in Figure 4.4D.



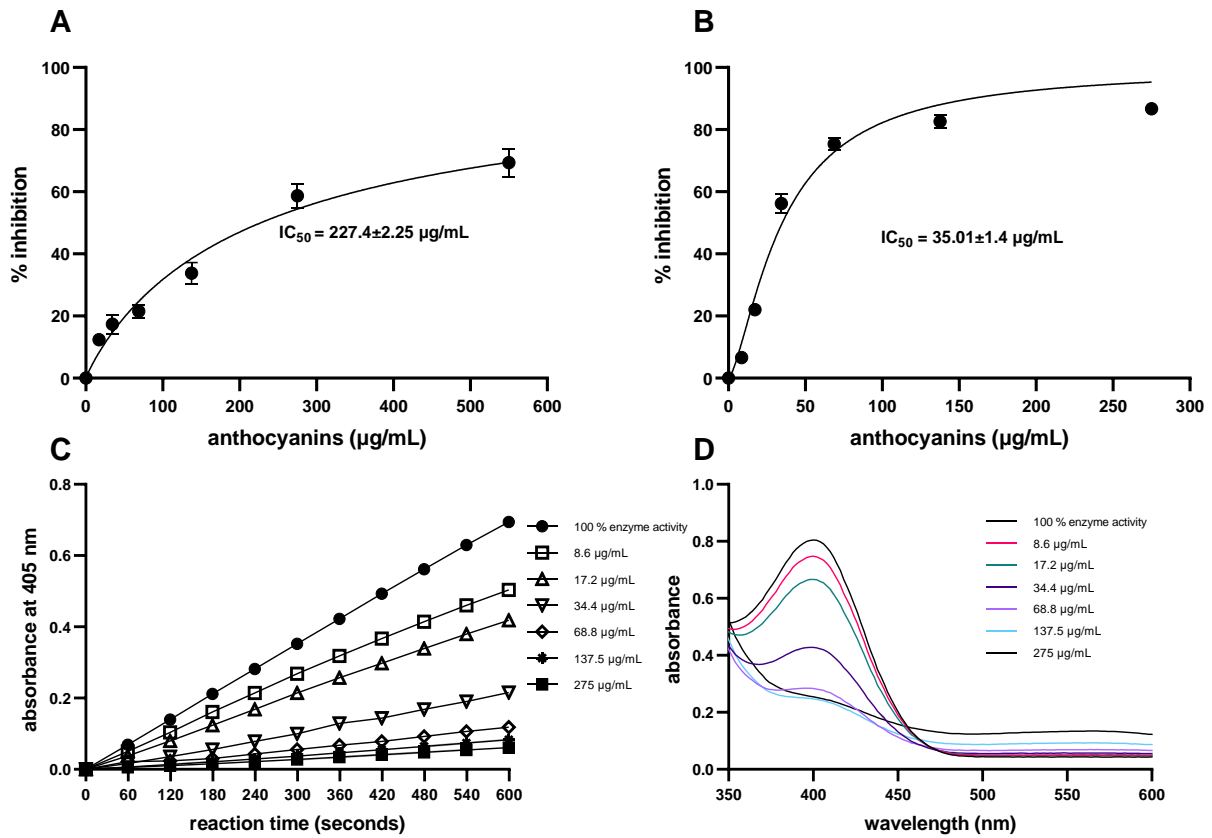
**Figure 4.3** Reaction progress curves at different concentration of substrate (using 1 U/mL of PPA) indication linearity of the reaction (A) and the Lineweaver-Burk double reciprocal plot (B).



**Figure 4.4** Dose-dependent effect of acarbose on inhibition of  $\alpha$ -amylase determined by (A) DNS assay using amylose as substrate and (B) by direct chromogenic assay using CNPG3 as a substrate. The kinetic data of the measurement (C) and wavelength scan (D) demonstrate the linear relationship of absorbance with substrate cleavage/product formation at 405 nm over time, and nitrophenol product formation at different concentrations of acarbose, respectively.

### 4.6.3 Enzyme inhibitory effects of anthocyanin containing extracts in DNS and chromogenic assay

The anthocyanins containing samples such as black currant, blueberry, cherry, pomegranate, and hibiscus were analyzed under both assay conditions for their enzyme inhibitory properties. The blackcurrant sample, which consists of anthocyanins only (>95% purity), inhibited enzyme activity in both assays, with  $IC_{50}$  227  $\mu$ g anthocyanins/mL and  $IC_{50}$  35  $\mu$ g/mL for the DNS and direct chromogenic assay, respectively (Figure 4.5 A & B). Similar to acarbose, the kinetic measurement (Figure 4.5C) and wavelength scan (Figure 4.5D) of blackcurrant demonstrated a linear response ( $r > 0.99$ ) and identity of nitrophenol release in the presence of different concentrations of blackcurrant. The results of other fruits extracts indicated that the DNS assay data did not support inhibitory properties for these extracts, even though SPE was employed to remove potentially interfering anthocyanins (Nyambe-Silavwe et al., 2015). Furthermore, it has been reported that the efficiency of the DNS assay may be compromised by the presence of intrinsic sugars (glucose, fructose), e.g. in fruit extracts, which may react with the DNS reagent and generate higher background absorbance. Indeed, the sugar content in all samples was >500 mg/mL, but very low in hibiscus (Table 4.2). The chromogenic assay, in contrast, indicated strong  $\alpha$ -amylase inhibition for pomegranate followed by blueberry extract, whereas weak and no inhibition was found for cherry and hibiscus, respectively (Table 4.3). Cyanidin and its glycosides are natural dietary pigments, which represent one of the major groups of naturally occurring anthocyanins. In the present study, cyanidin and a glycoside were tested in both assays. Whilst the DNS assay indicated only slight inhibition (7%) at concentrations up to 435  $\mu$ M for cyanidin, an  $IC_{50}$  of 141  $\mu$ M could be established using chromogenic assay. In contrast, cyanidin-3-O-galactopyranoside showed three times higher  $IC_{50}$  value with the same assay (414  $\mu$ M) (Table 4.3).



**Figure 4.5** Dose-dependent  $\alpha$ -amylase inhibition by blackcurrant determined by (A) DNS assay (0-1000  $\mu\text{g/mL}$ ) and (B) direct chromogenic assay (0-500  $\mu\text{g/mL}$ ). Substrate cleavage and product formation were monitored through kinetic measurement recording (B) and wavelength scan (C).

**Table 4.3 Comparison of  $\alpha$ -amylase inhibitory properties in anthocyanin-rich extracts and individual anthocyanins using direct chromogenic assay versus DNS assay**

Compounds/extract	$\alpha$ -amylase (%inhibition/ IC <sub>50</sub> )		$\alpha$ -glucosidase (%inhibition/ IC <sub>50</sub> )	Predominant anthocyanins
	DNS assay	Chromogenic assay		
<b>Black currant *</b>	227.4±2.3 $\mu$ g/mL	35.01±1.4 $\mu$ g/mL	3.13±2.1 $\mu$ g/mL	Dp3rut, Cy3rut, Dp3glu, Cy3glu (Farooque et al., 2018)
<b>Blueberry</b>	NO (645 $\mu$ g/mL)	80.44±2.0 $\mu$ g/mL	51.82±1.6 $\mu$ g/mL	Dp3glu, Mv3glu, Dp3glc, Mv3glc (Bunea et al., 2013)
<b>Cherry</b>	NO (at 536 $\mu$ g/mL)	30 % at 268 $\mu$ g/mL	22±1.9 $\mu$ g/mL	Cy3rut, Cy3glu (Serrano et al., 2005)
<b>Pomegranate</b>	NO (at 31 $\mu$ g/mL)	11.33±2.3 $\mu$ g/mL	0.1±2.3 $\mu$ g/mL	Dp3,5 diglu, Cy3,5 diglu, Dp3glu (Mousavinejad et al., 2009)
<b>Hibiscus</b>	NO (at 218 $\mu$ g/mL)	NO (at 218 $\mu$ g/mL)	69.42±1.5 $\mu$ g/mL	Dp3sam, Cy3sam (Iffe et al., 2016)
<b>Mahaleb cherry (pure)*</b>	ND	5 % (at 34 $\mu$ g/mL)	7.2 % (at 34 $\mu$ g/mL) °	Cy3glu (Blando et al., 2018)
<b>Mahaleb cherry (crude)</b>	ND	21 % (at 39 $\mu$ g/mL)	48.3 % (at 39 $\mu$ g/mL) °	Cy3glu (Blando et al., 2018)
<b>Black carrot (pure)*</b>	ND	NO (at 57.7 $\mu$ g/mL)	7.3 % (at 57.5 $\mu$ g/mL) °	Cy3glc (Blando et al., 2018)
<b>Black carrot (crude)</b>	ND	NO (at 48 $\mu$ g/mL)	25.6 % (at 48 $\mu$ g/mL) °	Cy3glc (Blando et al., 2018)
<b>Cyanidin</b>	7 %	141±1.6 $\mu$ g/mL (491 $\mu$ M)	15.42±1.6 $\mu$ g/mL (4.43 $\mu$ M)	
<b>Cyanidin-3-O-Galactospyranoside</b>	ND	414±2.6 $\mu$ g/mL (845 $\mu$ M)	361±1.3 $\mu$ g/mL (745 $\mu$ M)	

Data are expressed as mean with SEM of three replicates. Results of % inhibition are calculated to total anthocyanin content. Abbreviations are as follows: Dp3rut, delphinidin-3-O-rutinoside; Cy3rut, cyanidin-3-O-rutinoside; Dp3glu, delphinidin-3-O-glucoside; Cy3glu, cyanidin-3-O-glucoside; Dp3glc, delphinidin-3-O-galactoside; Mv3glu, malvidin-3-O-glucoside; Mv3glc, malvidin-3-O-galactoside; Dp3,5 diglu, delphinidin 3,5 diglucoside; Cy3,5 diglu, cyanidin 3,5 diglucoside; Dp3sam, delphindin-3-O-sambubioside; Cy3sam, cyanidin-3-O-sambubioside; cyanidin-3-O-galactoside; Cy3glc, Cyanidin-3-O-galactospyranoside; ND, not determined by DNS assay; NO, no inhibition was found.

\* purified sample containing >95% anthocyanins

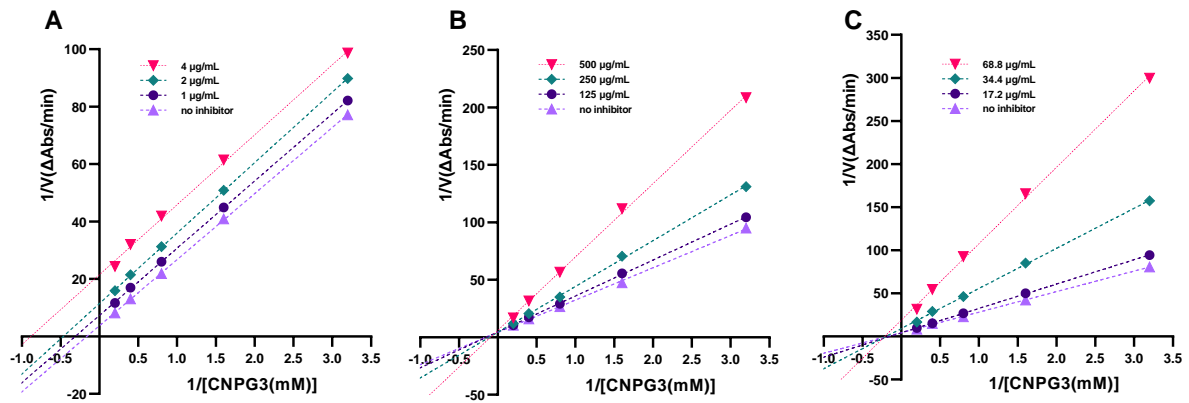
#### **4.6.4 Application of chromogenic assay to determine $\alpha$ -amylase inhibition in crude and pure extracts**

The present study further investigated the contribution of anthocyanins to enzyme inhibition. Crude extracts from mahaleb cherry and black carrot and corresponding purified anthocyanin fractions were analyzed. These samples have been reported for their antioxidant, anti-inflammatory and cardiovascular-protective properties (Blando et al., 2018). As Table 4.3 indicates, there were only weak  $\alpha$ -amylase inhibition properties of mahaleb cherry at indicated concentrations and no inhibitory effect was evident from black carrot, despite the fact that the predominant anthocyanin is cyanidin-3-galactoside (Blando et al., 2018). The current study also investigated, apart from cyanidin and cyanidin-3-galactopyranoside, the contribution of anthocyanin metabolites to amylase inhibition, i.e. gallic acid, chlorogenic acid, and protocatechuic acid. Even at high concentrations of 1000  $\mu\text{g/mL}$  there was no inhibition of  $\alpha$ -amylase for any of these compounds.

#### **4.6.5 Mode of inhibition of anthocyanins on $\alpha$ -amylase activity**

While  $\text{IC}_{50}$  values showed the potency of natural compounds towards enzyme inhibition, further valuable information was obtained by determining the kinetics of inhibition by group or individual compounds isolated from natural extracts. An inhibitor can interact with an enzyme in various ways. Studies on the kinetics of inhibition are major tools that enable to distinguish between different inhibitory mechanisms. The initial velocity 'V' of the hydrolysis reactions catalyzed by pancreatic  $\alpha$ -amylase was measured at various substrate concentrations [S] (0–5 mM) in the presence/absence of inhibitors. The results of Lineweaver-Burk plots, shown in Figure 4.6, demonstrate significant changes in  $K_m$  and  $V_{\text{max}}$  in the presence or absence of inhibitors. In the presence of acarbose (1, 2, 4  $\mu\text{g/mL}$ ), both  $V_{\text{max}}$  and  $K_m$  decreased, indicating uncompetitive enzyme inhibition (Figure 4.6A). With cyanidin-3-O-galactopyranoside in the reaction mixture (125, 250, and 500  $\mu\text{g/mL}$ ),  $V_{\text{max}}$  values remained constant whereas  $K_m$  values changed to 7.95, 10.79, 23.46 mM, respectively (Figure 4.6B), indicating that cyanidin-3-O-galactopyranoside is a competitive inhibitor. In contrast, with

blackcurrant anthocyanins, the  $K_m$  remained unaffected whereas  $V_{max}$  decreased, showing mixed type inhibition of  $\alpha$ -amylase (Figure 4.6C).



**Figure 4.6** Lineweaver–Burk plots demonstrating inhibition type of (A) acarbose, (B) cyanidin-3-O-galactoglucoside and (C) blackcurrant against pancreatic  $\alpha$ -amylase.

#### **4.6.6 $\alpha$ -glucosidase inhibition by anthocyanin containing extracts and pure compounds**

In addition to  $\alpha$ -amylase, the present study also determined the inhibitory potential of anthocyanin-rich fruits extracts and pure compounds against  $\alpha$ -glucosidase. In general, the anthocyanins containing samples/pure anthocyanins are stronger inhibitors of  $\alpha$ -glucosidase than  $\alpha$ -amylase (Table 4.3). Moreover, the extracts with highest polyphenolic contents (Table 4.1) such as pomegranate exhibited more inhibitory activities than all the other tested samples.

### **4.7 Discussion**

The intake of anthocyanin-rich extracts or foods such as blackcurrant, cranberry juice, and mixed berries has been shown to reduce postprandial glycaemia, and insulinemia (Wilson et al., 2008; Torronen et al., 2012; Castro-Acosta et al., 2016). The most likely and quantitatively most relevant mechanism by which fruit extracts reduce postprandial hyperglycaemia is the attenuation of starch breakdown through  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition (Akkarachiyasit et al., 2011; Adisakwattana et al., 2012; Barik et al., 2020). There is a huge interest in assessing  $\alpha$ -amylase inhibitory properties of plant bioactives, however, the commonly used DNS assay is laborious and has shown interference with some compounds. Therefore, in the present study, a working protocol for the kinetic measurement of  $\alpha$ -amylase inhibition using a short chain substrate i.e., 2-chloro-4 nitrophenyl  $\alpha$ -D-maltotrioxide (CNP3) was successfully established using positive control (acarbose) and compared to DNS assay. These results of  $\alpha$ -amylase inhibitory potential of acarbose are in line with other literature reporting using DNS assay (

Table 4.4). The outcomes of the direct chromogenic assay are in agreement with previous research (Table 4.5) demonstrating acarbose-inhibition of PPA using chromogenic assay (Kalita et al., 2018; Freitas & Le Feunteun, 2019; Berger et al., 2020; Khadayat et al., 2020).

It has been reported that anthocyanins such as cyanidin-3-glucoside, cyanidin-3,5-glucoside, cyanidin-3-rutinoside, and peonidin-3-glucoside exert *in vitro* inhibition towards  $\alpha$ -amylase

(Sui et al., 2016). For example, cyanidin-3-rutinoside, a major anthocyanin found in many fruits such as sweet cherry and blackcurrant, is a strong  $\alpha$ -amylase inhibitor ( $IC_{50}$  24.4  $\mu$ M) (Akkarachiyasit et al., 2011). Previously, blackcurrant (puree) inhibited  $\alpha$ -amylase activity with an  $IC_{50}$  value of 501  $\mu$ g/mL, using the same substrate (CNP3G), however, given the high acarbose  $IC_{50}$  value (441  $\mu$ g/mL, Table 4.5) stated by this group, these data may not be directly comparable (Berger et al., 2020). The blackcurrant anthocyanin extract used in the present study contained around 45% delphinidin-3-O-rutinoside, 31% cyanidin-3-O-rutinoside as major anthocyanins, and the corresponding glucosides at 16% and 8%, respectively, as minor anthocyanins (Farooque et al., 2018). Enzyme inhibitory properties are known for a number of polyphenols. In fact, the presence of other polyphenols in extract samples may have contributed to inhibitory activities. As Table 4.1 indicates, there are marked differences in total polyphenol and anthocyanin concentration of different samples with pomegranate having the highest amount of total polyphenols which may therefore likely have contributed to its lower  $IC_{50}$  value. The lower  $IC_{50}$  value of cyanidin compared to cyanidin-3-O-galactopyranoside are in alignment with the literature demonstrating that increasing numbers of sugar moieties attached to the anthocyanin molecule reduce inhibitory activity. For example, cyanidin-3-glucoside demonstrated higher inhibitory activity compared to cyanidin-3,5-diglucoside (Akkarachiyasit et al., 2010). Further, previous reports indicated an  $IC_{50}$  of 380  $\mu$ M for PPA inhibition by cyanidin using DNS assay and starch as substrate (Akkarachiyasit et al., 2010), but no inhibitory effects were observed by others when synthetic substrate was applied (Berger et al., 2020). The results of present study demonstrated increased detectability (5-10 fold) of  $\alpha$ -amylase inhibitory properties for the positive control acarbose as well as anthocyanins and anthocyanins-rich extracts with the direct chromogenic assay in comparison to the DNS assay. The reason for the discrepancy is likely related to differing substrate properties (Dona et al., 2010). For example, amylose and amylopectin, showed under the same assay conditions  $IC_{50}$  values of 3.5  $\mu$ M and 10  $\mu$ M (Nyambe-Silavwe et al., 2015), respectively, emphasizing the link between increased substrate complexity and enzyme activity. Indeed, when using potato starch as a substrate instead of amylose, the  $IC_{50}$  values

of acarbose increased from 58.3 to 254  $\mu\text{M}$  in our laboratory. In contrast, CNPG3 consists of a tri-glucoside linked to a chromogen, a short chain and linear molecule that may be well accessible for  $\alpha$ -amylase, but actually may have less affinity to the enzyme compared to a longer substrate (Visvanathan et al., 2021)

**Table 4.4 Summary of studies measuring  $\alpha$ -amylase inhibition by pure anthocyanins and anthocyanin-rich samples via DNS assay**

Inhibitor	Substrate	Enzyme (PPA)	IC <sub>50</sub> ( $\mu\text{g/mL}$ )	Buffer (pH)	Incubation time	References
Acarbose	Starch	1% 3 U/mL	77.47	Phosphate (6.9)	10 min	Akkarachiyasit et al. (2010)
Cyanidin	(w/v)		109.15			
Cya-3-glucoside			145.45			
Cya-3-galactoside			> 450			
Cya-3,5-diglucoside			> 600			
Acarbose	Starch	1% 3 U/mL	11.69	Phosphate (6.9)	10 min	Akkarachiyasit et al. (2011)
Cya-3-rutinoside	(w/v)		15.53			
Cya-3-glucoside	Starch	1% Not clear	11.64	Phosphate (6.9)	15 min	Sui et al. (2016)
Cya-3,5-glucoside	(w/v)		24.46			
Cya-3-rutinoside			18.46			
Peo-3-glucoside			34.76			
Coffee anthocyanins	Starch	1% 0.5 mg/mL	430	Phosphate (6.9)	10 min	Murthy et al. (2012)
	(w/v)					
Whortleberry	Starch	0.5 % 0.5 U/mL	1910	Phosphate (6.9)	15 min	Amin (2010)
Mal-3-glucoside	(w/v)		162.34			

**Table 4.5 Summary of studies measuring  $\alpha$ -amylase inhibition by pure anthocyanins and anthocyanin-rich samples via direct chromogenic assay**

<b>Inhibitor</b>	<b>Substrate</b>	<b>Enzyme</b>	<b>IC<sub>50</sub> (<math>\mu</math>g/mL)</b>	<b>Buffer (pH)</b>	<b>Incubation time</b>	<b>References</b>
Acarbose	CNPG3	1.5 U/mL PPA	6.1	PBS (7) with 0.9% NaCl	10 min	Khadayat et al. (2020)
Acarbose <b>Berry juices</b> Aronia, Bilberry, Sour cherry <b>Berry concentrates</b> Aronia, Lingonberry <b>Berry purees</b> Blackcurrant, Bilberry, Cranberry	CNPG3 4mM	30 U/mL PPA	441 273, 1088, 1943 381, 361	PBS (6.9) 40 mM	10 min	Berger et al. (2020)
Acarbose Red potato Purple potato	CNPG3 2mM	1 $\mu$ g/mL PPA	12.86 25.52 30.82	PBS (6.5) 50 mM Containing 200 mM NaCl, 5 mM CaCl <sub>2</sub>	10 min	Kalita et al. (2018)
Cya-3-O-glucoside Cya-3-rutinoside Mal-3-O-glucoside Mal-3,5-O-diglucoside	CNPG3 (not clear)	2 nM HSA	180 200 675 80	MES (6)	Not clear	Homoki et al. (2016)
Acarbose Pel-3-rutinoside Peo-3-arabinoside Peo-3-galactoside Mal-3-arabinoside	CNPG3 2mM	0.5 U/mL HPA	0.47 26.54 52.65 43.59 39	PBS (7) 0.05 mM	30 min	Xie et al. (2020)

**Abbreviations:** CNPG3, 2-chloro-4-nitrophenol  $\alpha$ -D-Maltotrioside; HSA, human salivary amylase; HPA; human pancreatic amylase; PPA, porcine pancreatic amylase; PBS, phosphate buffer solution; Cya, cyanidin; Mal, malvidin; Pel, pelargonidin; Peo, peonidin.

Inhibitory effects towards  $\alpha$ -amylase have been related to anthocyanins but may also be due to other compounds. Indeed, in the present study,  $\alpha$ -amylase-inhibitory properties were found to be independent of anthocyanin content, i.e. pomegranate, which has a low anthocyanin content (Table 4.1), has demonstrated strongest inhibitory properties among the berry samples (Table 4.3). Therefore, the presence of other polyphenolic compounds in fruit concentrates might be responsible for the differing inhibitory potential against  $\alpha$ -amylase, which, in case of pomegranate, might be due to ellagitannins, especially punicalin and punicalagin (Bellesia et al., 2015). Similar findings were also reported for strawberry and raspberry extracts (McDougall et al., 2005). In contrast, Grussu et al. (2011) has recently emphasized that ellagitannins might not be solely responsible for amylase inhibition. Indeed, it was observed that both yellow raspberries and red raspberries extracts inhibited  $\alpha$ -amylase to the same extent, questioning further the contribution of anthocyanins. Results of the present study i.e. blackcurrant-derived anthocyanins, however, confirm the potential of anthocyanins to inhibit  $\alpha$ -amylase enzyme. Previously, it has been reported that anthocyanins are stronger inhibitors of  $\alpha$ -glucosidase than  $\alpha$ -amylase (McDougall et al., 2005; Berger et al., 2020). The results (Table 4.3) of the present study are in line with literature showing more inhibition of  $\alpha$ -glucosidase compared to  $\alpha$ -amylase by anthocyanin-rich samples as well as by isolated compounds. Similar to  $\alpha$ -amylase inhibition, the extent of  $\alpha$ -glucosidase inhibition also varies among different fruit extracts. It can therefore be assumed that differently structured anthocyanins (e.g. from different sources) may contribute differently to enzyme inhibition. For example, increased inhibitory activities were observed for anthocyanins with more hydroxyl groups (Tadera et al., 2006; Berger et al., 2020). Indeed, it has been shown that delphinidin (three hydroxyl groups on the B ring) inhibited digestive enzymes more efficiently than cyanidin and petunidin which have two hydroxyl moieties (Promyos et al., 2020).

The present study indicated competitive and mixed type of inhibition by isolated anthocyanins and purified anthocyanins containing sample. The results are consistent with a previous study suggested that purified anthocyanins competitively inhibited the hydrolysis of synthetic

substrate by human salivary amylase (Homoki et al., 2016). Another study demonstrated that anthocyanins such as cyanidin-3-glucoside, cyanidin-3,5-glucoside, cyanidin-3-rutinoside, and peonidin-3-glucoside inhibited the activity of , porcine pancreatic  $\alpha$ -amylase competitively measured via DNS method (Sui et al., 2016). In competitive inhibition, the inhibitor and the substrate compete with each other for the active site of enzyme suggesting that anthocyanins might compete with the CNPG3 in the present study and exert their inhibitory effect via binding with active site of  $\alpha$ -amylase. Previously, mixed-type inhibition (competitive and non-competitive) was observed for anthocyanin-rich bilberry extract against PPA measured by DNS method, suggesting that inhibitors are not only bound to enzymes, but they are also bound with enzyme-substrate complexes to create ternary inhibitor-enzyme-substrate complexes resulting in decreased enzyme activity (Ji et al., 2021).

The  $K_m$  value (Michaelis constant) indicates the affinity of an enzyme for a substrate. The lower the  $K_m$  value, the higher the affinity of the enzyme for the substrate. Compared to native starch, short chain *p*-nitrophenyl-linked maltose derivatives such as CNPG3 exhibit a lower affinity for amylase (Slaughter et al., 2001; Visvanathan et al., 2020; Visvanathan et al., 2021). The  $K_m$  value (4.4 mg/mL) for CNPG3 observed in the present study was higher than for the maize starch (0.73 mg/mL) (Ji et al., 2021), indicating less affinity of CNPG3 for  $\alpha$ -amylase. Similarly, Nyambe-Silavwe et al. (2015) reported lower  $K_m$  value for amylose (4.5 mg/mL) compared to amylopectin (12 mg/mL) at substrate concentration of 4.5 mg/mL.

## 4.8 Conclusion

One of the disadvantages of the DNS method is that it does not give a direct estimation of the reaction product. On the other hand, CNPG3 is effectively cleaved by  $\alpha$ -amylase at the dye-glycoside bond generating *p*-nitrophenol, a yellow coloured compound with wavelength maximum at 405 nm. The intensity of *p*-nitrophenol is proportional to CNPG3 hydrolysis, with the increased absorbance corresponding directly linked to  $\alpha$ -amylase activity. The accuracy and reliability of the DNS assay is also impacted by the interference caused by pigmented samples and non-equivalence between the amount of sugar reacted and DNS produced

(Visvanathan et al., 2021). In comparison, the kinetic measurement of  $\alpha$ -amylase activity using CNPG3 rather than endpoint measurement and recording of absorbance/wavelength scan at the end of reaction time confirm that the direct chromogenic assay is not impacted by intrinsic sample colour or background signals of pigmented samples and therefore well suitable for measuring  $\alpha$ -amylase inhibitory properties of anthocyanins and others. Furthermore, reproducibility, accuracy, uniformity and reliability are some advantages of the microplate based direct chromogenic assay (Khadayat et al., 2020). A limitation of the direct chromogenic assay is due to the short chain substrate not representing the natural starch substrate exactly which may therefore not accurately reflect activity of  $\alpha$ -amylase (as compared to starch, the natural substrate for  $\alpha$ -amylase) (Visvanathan et al., 2020). Nevertheless, with the DNS assay, there is involvement of several steps such as adding enzyme/substrates/inhibitors into the reaction tubes, heating and cooling steps, transferring reaction mixture from reaction tubes to microplate and passing mixture through the cartridges make the DNS assay time-consuming especially when large numbers of samples need to be analysed. In contrast, in the direct chromogenic assay, after incubating enzyme with inhibitor, substrate is added, and absorbance is measured immediately up to 10 min. With the chromogenic assay being quick and easy to perform, this method facilitates rapid screening of a large number of samples.

In summary, the performance of the commonly used DNS assay was compared with a direct chromogenic assay to compare their performance when measuring pigmented samples. Given the lower  $IC_{50}$  with chromogenic assay and the easier controlled conditions, this method should be favoured for enzyme inhibition measurements in pigmented samples, in particular for screening purposes, if large numbers of samples are to be measured.

## Chapter 5

### ***In vitro* and *in vivo* antidiabetic properties of blueberry concentrate- a dose dependent effect/response**

#### **5.1 Abstract**

Consumption of carbohydrate-rich foods increases the postprandial glucose concentrations leading to hyperglycaemia, a risk factor associated with diabetes mellitus. Previous studies have suggested that anthocyanins may beneficially influence digestion/and absorption of carbohydrates and thereby suppress the postprandial hyperglycaemia, but the evidence are limited in terms of source and dose effects. The present study investigated the dose dependent effects of anthocyanin-rich blueberry juice on postprandial glycaemia. Nine healthy subjects (7 F/2 M) with normal fasting plasma glucose were enrolled in a randomised, controlled trial. Two doses of blueberry providing 327 mg (D1) and 545 mg (D2) total anthocyanins, or a control drink, matched with sugar against the highest blueberry dose were administered with white bread to provide in total 50 g available carbohydrates. Plasma glucose was measured in regular intervals over 180 min. To determine the contribution of blueberry on the activity of carbohydrate digesting enzymes as a potential mechanism for its hypoglycaemic effect, *in vitro* inhibitory effects of blueberry on the activity of  $\alpha$ -amylase and  $\alpha$ -glucosidase were determined. The *in vivo* results indicated a significant reduction in peak postprandial glucose levels at 30 min following D1 ( $P < 0.002$ ) and D2 ( $P < 0.001$ ) blueberry drinks. Furthermore, total iAUC was also significantly reduced ( $P < 0.05$ ) after intake of both blueberry drinks. The activity of  $\alpha$ -glucosidase and  $\alpha$ -amylase was reduced dose dependently by blueberry with  $IC_{50}$  values of  $76.15 \pm 2.5 \mu\text{g}$  and  $260 \pm 3.2 \mu\text{g}$  polyphenol/mL, respectively. These findings confirmed that the beneficial effects of blueberry ingestion on postprandial glycaemia, at least partially due to inhibition of carbohydrate digesting enzymes. Blueberry consumption should therefore be encouraged in order to prevent and manage the T2DM.

## 5.2 Introduction

It is estimated that almost 463 million people are suffering from diabetes worldwide due to pancreatic  $\beta$ -cell dysfunction and/ or increased resistance to insulin with impaired glucose tolerance.(Alzaid et al., 2013; Saeedi et al., 2019). Diabetes leads to other complications, such as heart disease, renal function recession, and blindness (Deshpande et al., 2008). Regular intake of high-carbohydrate diets may contribute to postprandial hyperglycaemia, a major risk factor in the development of T2DM (Zhang et al., 2011; Zhang et al., 2017). One of the most important mechanisms to prevent hyperglycaemia and subsequent diabetes is to control postprandial blood glucose concentrations. Hydrolysis of starch is initiated in the mouth by salivary amylase which convert complex polysaccharides to smaller units such as maltose. The last stage of starch digestion occurs in the small intestine, where pancreatic  $\alpha$ -amylase and brush border glucosidase enzymes complete amylolysis, and the ultimate product of this process, glucose, is finally absorbed into the bloodstream. Suppressing the activity of intestinal  $\alpha$ -glucosidase and pancreatic  $\alpha$ -amylase leads to reduction of starch hydrolysis (Turina et al., 2006), resulting in potentially reduced glucose available for absorption which is considered an effective strategy in the management and treatment of T2DM (Hanhineva et al., 2010).

Dietary habits and lifestyle are major factors influencing the onset and progression of T2DM. Regular intake of fruits and vegetables can prevent the risk of T2DM (Wang et al., 2016). It will reduce the need for pharmacological treatment, which is typically associated with side effects, in addition to being extremely expensive (Hanhineva et al., 2010). Acarbose, miglitol, and voglibose, which are currently used antihyperglycaemic drugs in the treatment of diabetes, prevent its progression mainly by inhibiting carbohydrate digestion, thereby reducing blood glucose levels (Raptis & Dimitriadis, 2001). However, these synthetic inhibitors can have side effects (such as nausea, abdominal pain, flatulence), leading to an interest in exploring natural sources as possible alternatives. In recent years, flavonoids have been highlighted as potential  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors, as an alternative to acarbose (Tadera et al., 2006). *In vivo* animal study demonstrated that flavonoids, in particular anthocyanins, reduce

postprandial glycaemia by suppressing the gastrointestinal glucose transport and  $\alpha$ -glucosidase activity (Hanamura et al., 2006). Additionally, epidemiological data supports an association between anthocyanin consumption and a decrease in the incidence of T2DM and increased insulin sensitivity (Wedick et al., 2012; Jennings et al., 2014). Anthocyanins are naturally occurring pigments found in many fruits and vegetables including berries, cherries, grapes, red onion, red radish, and purple potatoes (Andersen & Jordheim, 2010). Cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin are the most predominant anthocyanins found in berries and have been reported for their antioxidant, anticancer, anti-obesity, anti-inflammatory, antidiabetic, and cardio-protective properties (Vendrame et al., 2016; Ma et al., 2018).

There is increasing evidence of reduced blood glucose elevations following anthocyanin-rich foods, but studies have reported mixed outcomes. For example, reduced postprandial glycaemia and insulinemia have been observed after ingestion of anthocyanin-rich blackcurrant (Castro-Acosta et al., 2016), cranberry juice (Wilson et al., 2008), mixed berry puree (Torrönen et al., 2010), and green tea with mixed freeze-dried fruit powders (Nyambe-Silavwe & Williamson, 2016). In contrast, some studies have reported no significant changes in postprandial blood glucose concentrations following fresh strawberries (Cao et al., 1998), cranberry juice (Vinson et al., 2008), and fresh blueberries and raspberries (Clegg et al., 2011) compared to control conditions.

Variations in postprandial outcomes may be explained by several factors, including, study design, anthocyanin dosages, administration methods (powder, juice, or in food product), presence of other bioactives, and choices of control food/beverage/carbohydrate source. Although the human trials have delivered mixed and inconsistent results, there are evidence supporting the antidiabetic properties of anthocyanins-rich foods. Among the berries, blueberries stand out for their anthocyanin content (400 to 500 mg/100 g) (Vendrame et al., 2016). Malvidin and delphinidin were the more abundant anthocyanins found in blueberry. Other polyphenolic compounds present in blueberry include flavanols (i.e., kaempferol,

quercetin, myricetin, phenolic acids (mainly hydroxycinnamic acids) and derivatives of stilbenes (Spinardi et al., 2019). Epidemiological evidence supports that blueberry consumption improved the insulin resistance in obese and insulin-resistant animals or humans (Stull, 2016). However, to date, human trials investigated the influence of anthocyanin-rich blueberry foods/beverages on glycaemic profiles and/or examined possible mechanisms are sparse (Stull, 2016; Vendrame et al., 2016).

### **5.3 Aims and objectives**

The aim of this study was therefore, to test the hypothesis whether anthocyanin-rich blueberry dose dependently reduces postprandial blood glucose concentrations in healthy adults. To identify potential underpinning mechanisms of action that exist *in vivo* for the regulation of postprandial glycaemia, the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzyme inhibitory properties of blueberry were determined *in vitro*.

### **5.4 Materials and methods**

#### **5.4.1 Reagents and samples**

Concentrated blueberry juice (BJ) was purchased from Holland and Barrett® (Leeds, UK). White bread (Warburtons®), household sugar and low nitrate (<0.1 mg/L) still natural Buxton® mineral water were all purchased from a local Tesco® store (Leeds, UK). Individual sugars (fructose, glucose, sucrose) were purchased from Holland and Barrett® (Leeds, UK). The details about all other chemicals and reagents used in this study are described in sections of Chapter 2 Materials and Methods.

#### **5.4.2 Characterization of blueberry samples (Total phenolic, monomeric anthocyanins and sugar content)**

The total polyphenols and total monomeric anthocyanins in BJ were determined using the Folin-Ciocalteu method and pH differential method, respectively, as described in section 2.2 in Chapter 2 Materials and Methods. Gallic acid (GA) was used as a reference standard ranging from (0 – 500  $\mu$ g/mL) to calculate polyphenols and results were expressed as mg/mL

GA equivalents. Total anthocyanins in BJ were calculated on the basis of most abundant anthocyanins present in blueberry. Soluble sugars (glucose, fructose, sucrose) present in blueberry concentrate were analysed by chromatographic technique HPLC-ELSD as described. Individual sugars were quantified using external standard curves of glucose, fructose and sucrose in the range of 250 - 3000 µg/mL with fucose being added as an internal standard.

### 5.4.3 *In vivo* human study

#### 5.4.3.1 Participant recruitment

The blueberry study was conducted as a part of an MSc project (Huda Alawi). The PhD student has been trained and supported the MSc student in establishing the *in vivo* postprandial glucose response experiments. Nine healthy participants (7 women/2 men), meeting the inclusion criteria (aged 20-30 years; average BMI  $\geq 24.9$  kgm<sup>-2</sup>, not pregnant or breast-feeding, no taking any medication or supplements) were recruited using advertisements, flyers and personal communications. The detailed information about study requirements is presented in section 3.6 in Chapter 2 Materials and methods.

**Table 5.1 Characteristics of the subjects at baseline**

	mean±SEM
<b>Ethnicity</b>	Mixed
<b>Gender</b>	2 male/7 female
<b>Age (years)</b>	27±0.93
<b>Height (cm)</b>	161±2.94
<b>Weight (kg)</b>	62.91±3.73
<b>Body mass index (kg/m<sup>2</sup>)</b>	23.4±0.81
<b>Fasting glucose (mmol/L)</b>	5.26±0.3
<b>Activity level</b>	Moderate

### 5.4.3.2 Preparation of test drinks

The low and high anthocyanin dose test drinks were prepared by diluting 30 mL and 50 mL of concentrated blueberry juice with water to 300 mL volume, respectively. Bottled water (300 mL) was used as a reference/control drink. To align the sugar content in the test drinks, the total sugars were adjusted to higher dose (29.4 g) of blueberry juice to achieve similar profile and amount of available carbohydrates for postprandial glucose response experiments as shown in Table 5.2.

**Table 5.2 Characteristics of tested meals/drinks**

Type of drink	Analysed sugars (g)			Total intrinsic sugars (g)	Available CH from bread (g)	Added sugars (g)		Total available CH (g)	Total PP (mg)	Total ACNs (mg)
	Fructose	Glucose	Sucrose			Fructose	Glucose			
Control	0	0	0	0	20.64	18.74	10.65	50	0	0
D1	11.25	6.38	ND	17.63	20.64	7.5	4.3	50	568.8	327
D2	18.74	10.65	ND	29.4	20.64	0	0	50	948	546

PP; polyphenols, ACNs; anthocyanins, CH; carbohydrates

### 5.4.3.3 Study design

Participants were subjected to intervention drinks randomly using an online programme (<http://www.randomization.com>). They attended the human study facilities at the University of Leeds, UK, on three mornings, separated by 2-3 days between each visit. On arrival, baseline blood glucose was measured, and subsequently, volunteers were asked to consume the blueberry drinks containing either 30 mL (D1) or 50 mL (D2) amount of BJ or a sugar matched control drink (300 mL water) alongside 45 g of bread within a few minutes (<5 min). Each drink provided 50 g available carbohydrates. Capillary blood was collected via finger prick in regular intervals for 3 h post consumption during each visit. Blood glucose levels were determined using glucometer Accu-Chek® Performa. Small volumes of blood (100 µL) were collected using microvettes at each time point. Samples were centrifuged at 1300 g, 4°C for 15 min and plasma aliquots were kept frozen at -80°C until insulin analysis. Samples were spoiled due to breakdown of freezer during the lockdown period (Covid 19). Therefore, insulin could not be measured in this study.

#### **5.4.3.4 Statistical analysis**

A minimum of ten subjects is generally considered sufficient to provide reasonable power and precision (Brouns et al., 2005). Based on a similar glycaemic study (Torronen et al., 2010), initially a total of eleven subjects were screened but only nine participants completed the study. The statistical analysis was performed using SPSS (version 26, IBM). The significance of the overall drink × time interaction and their main effects on blood glucose response was tested using a two factors repeated measure ANOVA and comparisons were conducted using Tukey's test, where a significant difference was observed. In addition, the maximum increase from baseline was calculated and the difference between the test drinks was analysed. The 0–180 min areas under the glucose response curve were calculated using GraphPad Prism; Version 9, ignoring the area below the baseline concentration, and the statistical significance was assessed with one way ANOVA. Values of  $P \leq 0.05$  were considered significant. Due to loss of plasma samples as mentioned earlier, insulin was not measured in this study.

#### **5.4.4 *In vitro* enzyme inhibition**

All the materials and methods used in the preparation of buffer solutions, enzyme and substrate solutions and to determine  $\alpha$ -amylase and  $\alpha$ -glucosidase activity are described in section 2.3 in Chapter 2 Materials and methods.

To make stock solution for enzyme assays, concentrated BJ was diluted (1:10) in Millipore water, centrifuged and supernatant was taken. The supernatant was further diluted in 20 mM phosphate buffer (pH 6.9) to provide a concentration range of 0 -1896  $\mu\text{g}/\text{mL}$  polyphenols for  $\alpha$ -amylase assay. The  $\alpha$ -amylase inhibitory properties of BJ were determined using direct chromogenic method using PPA and CNPG3 as a substrate.

For the  $\alpha$ -glucosidase assay, BJ supernatant was diluted in 0.1 M phosphate buffer (pH 7.0) to provide a concentration range of (0 - 237 mg polyphenols/mL) and the activity of (from yeast *S. cerevisiae*) was determined via absorbance assay using *p*-nitrophenyl- $\alpha$ -D-

glucopyranoside as a substrate. Acarbose, a synthetic inhibitor of  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes, was used as a positive control in both assays.

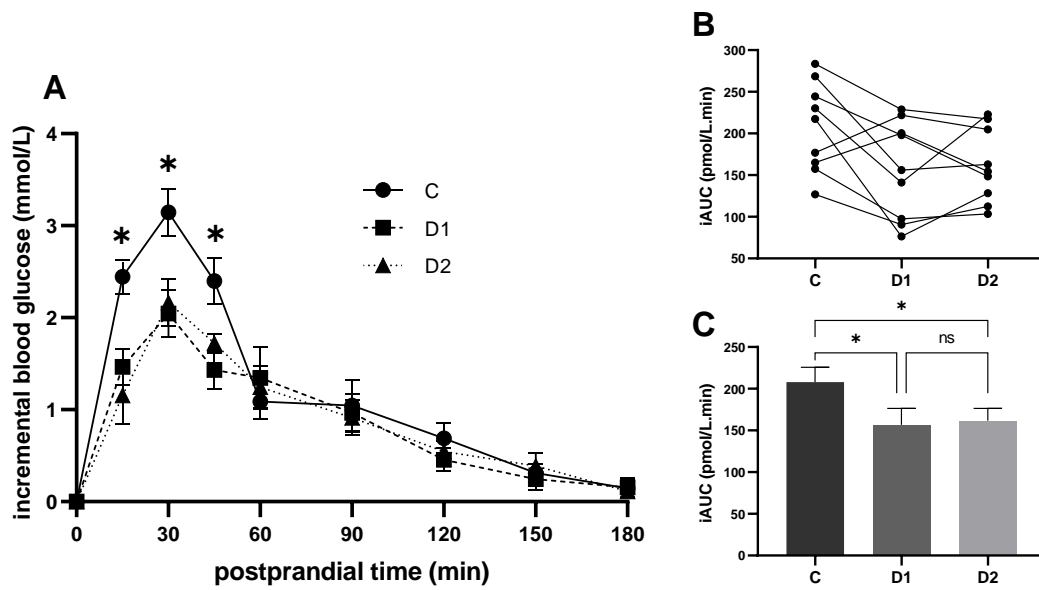
## **5.5 Results**

### **5.5.1 Compositional data of blueberry juice**

The results of compositional analysis showed that BJ contained  $18.96 \pm 0.21$  GAE mg/mL of total polyphenols, and  $10.9 \pm 1.12$  mg/mL of total anthocyanins. In addition, the HPLC-ELSD analysis for sugars demonstrated the presence of fructose (0.4 mg/mL) and glucose (0.213 mg/mL) in BJ, however, sucrose was not detected.

### **5.5.2 Effect of blueberry on postprandial plasma glucose response**

The mean incremental postprandial changes in blood glucose and total iAUC after consuming control/blueberry juice with a carbohydrate source (white bread) are shown in Figure 5.1. The meal  $\times$  time interaction for glucose response was highly significant ( $P = 0.001$ ). There was a significant difference ( $P < 0.05$ ) for both the iAUC and peak glucose concentrations between the control and blueberry drinks. The changes in individual iAUC after blueberry consumption are shown in Figure 5.1B. Post hoc analysis with Tukey's adjustment showed significantly lower glucose concentrations following low and high blueberry drink compared with control at 15-, 30-, and 45- post drink (Figure 5.1A). The low and high dose blueberry juice resulted in a decrease in the total glucose iAUC of  $-22.6 \pm 9.8\%$  and  $-20.2 \pm 6.69\%$  ( $P < 0.05$ ;  $n = 9$ ), respectively (Figure 5.1C). There was no significant difference ( $P = 0.961$ ) in total iAUC between the two test drinks. The maximum increase in plasma glucose concentration from the baseline was also smaller after the blueberry drinks (D1,  $2.3 \pm 0.23$  mmol/L;  $P = 0.001$  and D2,  $2.2 \pm 0.24$  mmol/L;  $P = 0.002$ ) intake than the control drink ( $3.3 \pm 0.20$  mmol/L). When analysed separately, no significant difference ( $P = 0.77$ ) was observed between the two blueberry test drinks.



**Figure 5.1** Postprandial blood glucose responses of 9 healthy participants after consumption of blueberry juice. Mean ( $\pm$  SEM) incremental changes in glucose concentrations (A) in response to equal amounts of carbohydrate from white wheat bread consumed with either sugar matched control (C), low dose (D1) or high dose (D2) of blueberry concentrate. Time points at which statistically significant differences were observed (two-way ANOVA; multiple comparison Tukey's test) are identified by \* symbol. Individual changes in iAUC (B) were also presented. The total iAUC was reduced significantly ( $P < 0.05$ ) by both doses of blueberry drink (C).

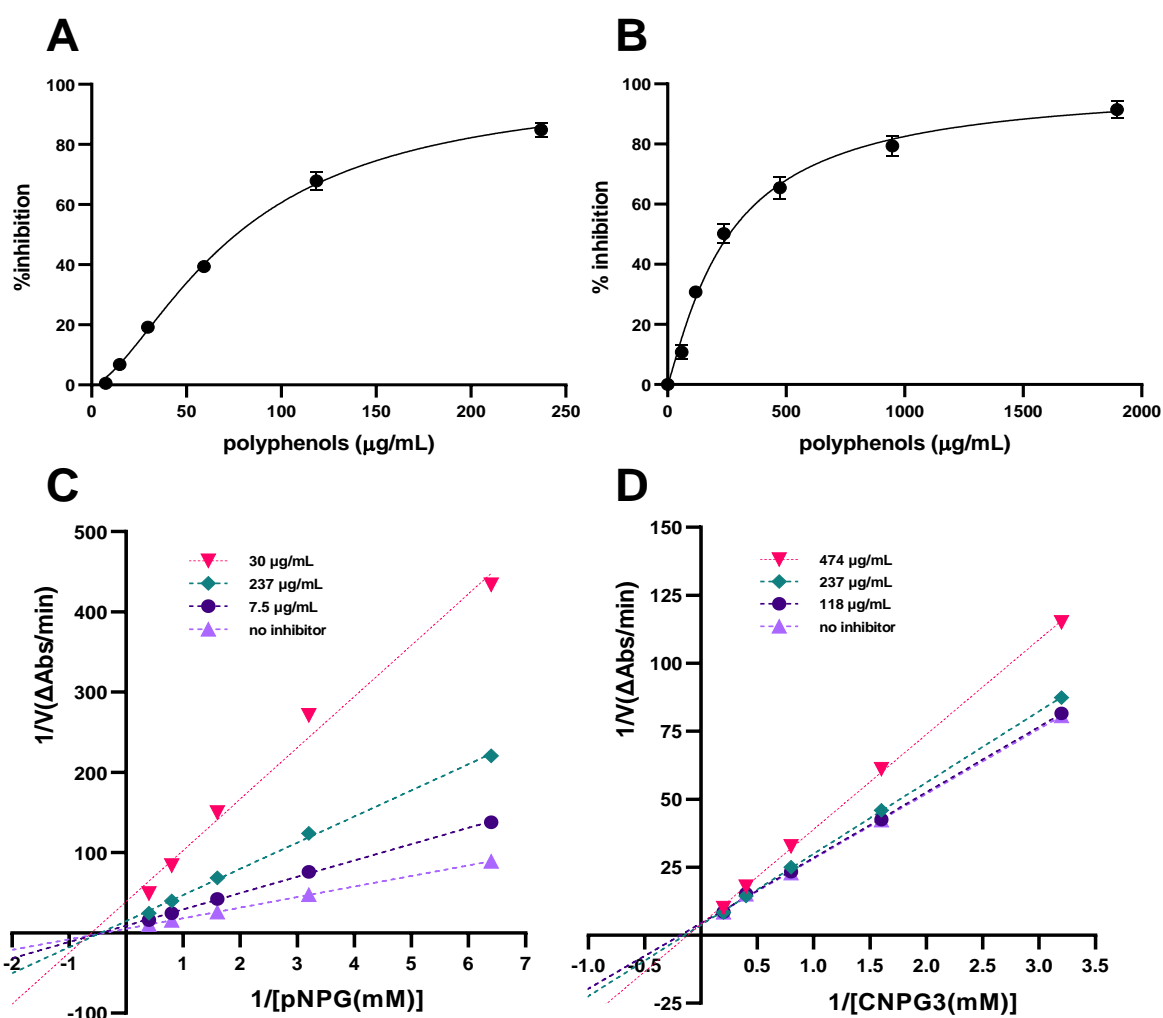
### **5.5.3 *In vitro* enzyme inhibition**

The enzyme inhibitory properties of blueberry juice were determined through the hydrolysis of synthetic substrates *p*-nitrophenyl glucopyranoside for  $\alpha$ -glucosidase and 2-chloro-4-nitrophenyl  $\alpha$ -D-maltotrioxide for  $\alpha$ -amylase. These short chain molecules released the yellow coloured *p*-nitrophenol upon enzymatic reaction at 37°C. The results of different dilutions of concentrated BJ on the activity of  $\alpha$ -glucosidase and pancreatic  $\alpha$ -amylase demonstrated dose-dependent inhibition of both enzymes (Figure 5.2).

The IC<sub>50</sub> values shown in

Table 5.3 showed that BJ inhibited the activity of  $\alpha$ -glucosidase more efficiently than the synthetic inhibitor acarbose, when compared at the same concentration. Unlike  $\alpha$ -glucosidase, the results of  $\alpha$ -amylase assay indicated blueberry as a less potent inhibitor of PPA compared to acarbose (

Table 5.3). The Lineweaver–Burk plots indicated mixed type inhibition of  $\alpha$ -glucosidase (Figure 5.2C) whereas competitive inhibition of  $\alpha$ -amylase (Figure 5.2B) by blueberry. The kinetic measurement of enzyme activity for acarbose and blueberry demonstrated linearity (correlation > 0.99) of reaction over time (10 min) and clearly reduced *p*-nitrophenol formation in the presence of different concentrations of inhibitors through wavelength scans in both assays. The results of BJ are presented in Figure 5.3.



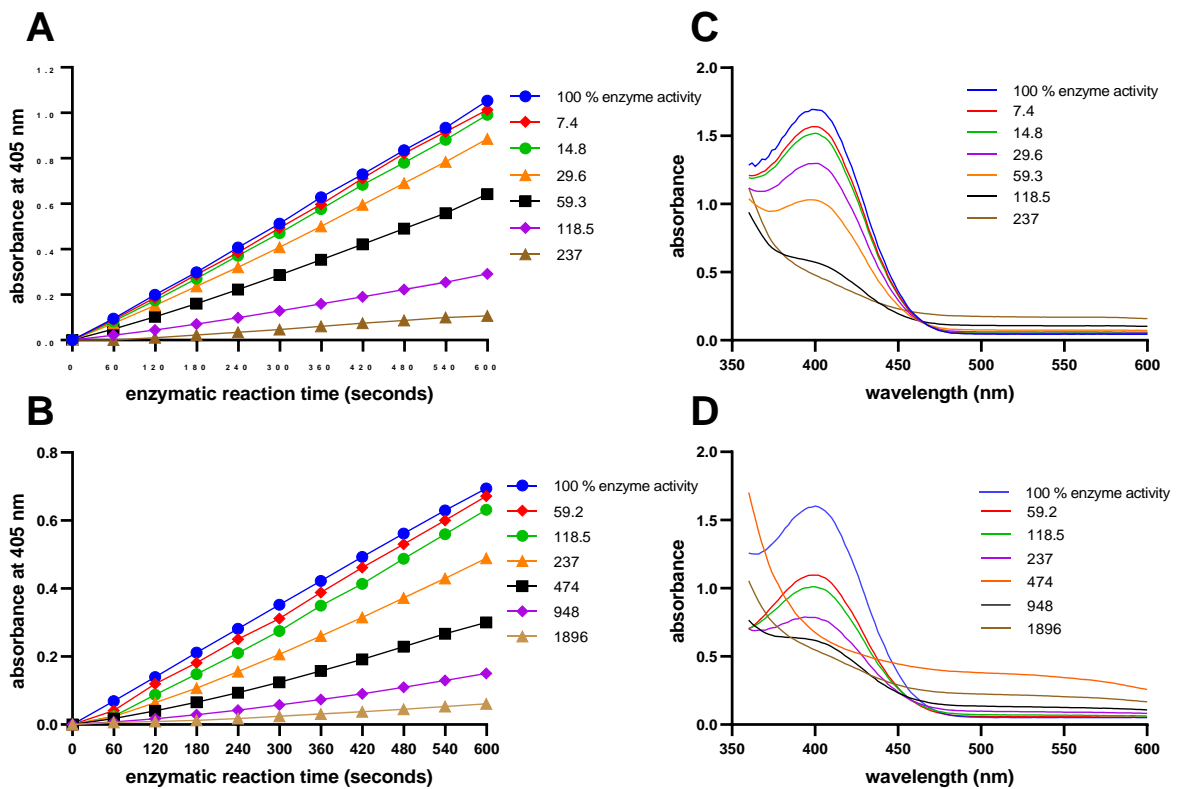
**Figure 5.2** The dose dependent inhibitory effects of BJ polyphenols on  $\alpha$ -glucosidase (A) and pancreatic  $\alpha$ -amylase (B). Lineweaver–Burk plots of  $\alpha$ -glucosidase (C) and  $\alpha$ -amylase (D). The results are expressed as means with SEM of three independent measurements performed in duplicate.



**Table 5.3** The IC<sub>50</sub> (µg/mL) values of blueberry against α-glucosidase, and pancreatic α-amylase

Samples	α-glucosidase	α-amylase
Acarbose	612.9±0.017	3.7±1.2
Blueberry	76.25±2.5	260±3.2

Results are expressed as mean±SEM, n=3



**Figure 5.3** The kinetic measurement and wavelength scan (350-600 nm) of α-glucosidase (A, C) and α-amylase (B, D) activity inhibition for blueberry. Shown is a representative set of data within one experiment.

## 5.6 Discussion

The acute blueberry consumption leads to a significantly ( $P < 0.05$ ) attenuated postprandial glycaemia in healthy population. The post-peak glucose increments were reduced by  $30 \pm 5.1\%$  and  $31 \pm 6.7\%$  after consumption of low and high dose anthocyanins blueberry at 30 min. However, no significant difference in postprandial plasma glucose concentrations ( $P > 0.05$ ) and total iAUC ( $P = 0.90$ ) was observed between two doses over the 3 h experimental period. The findings of the present study are consistent with previous research where anthocyanin-rich mixed berries meals/nectars (Torrönen et al., 2010; Torrönen et al., 2012; Torrönen et al., 2013), blackcurrant (Castro-Acosta et al., 2016), apple and blackcurrant (Castro-Acosta et al., 2017) consumed with carbohydrate source reduced postprandial hyperglycaemia. In contrast, a previous study (Bell et al., 2017) where two blueberry doses containing 310 and 724 mg anthocyanins, respectively, were administered in adults did not demonstrate any significant difference in peak plasma glucose concentrations after any of the blueberry dose ingestion as compared to sugar matched control drink. The reasons for this could be that the previous study did not involve any starchy food and test drinks were consumed after 2 h fasting period rather than 8-10 h as in the present study. The current study showed statistically significant ( $P < 0.005$ ) attenuation in peak plasma glucose during the first 45 min postprandial period (0-45 min) after blueberry consumption in comparison to control (Figure 5.1A).

Blueberries are rich in polyphenols such as chlorogenic acid, quercetin, kaempferol, myricetin, proanthocyanidins, catechin, epicatechin, resveratrol, and vitamin C which contribute to antioxidant activity. The total polyphenol content in blueberries ranges from 48 to 304 mg/100 g of fresh fruit weight. The major anthocyanins in blueberry are delphinidin and malvidin (Michalska & Lysiak, 2015). An increasing body of evidence suggests that blueberry reduced the risk of diseases such as cardiovascular disease (CVD), diabetes, obesity, and cognitive decline (Kalt et al., 2020). A chronic human trial involving 50 g carbohydrates replaced by 50 g blueberries per day demonstrated positive changes in metabolic factors such as body weight, insulin, cholesterol, and uric acid levels, in overweight young adults over 12 weeks

(Istek & Gurbuz, 2017). The structural variability may influence the magnitude of beneficial effects of anthocyanins *in vitro* and *in vivo*. The results (presented in Chapter 4) of *in vitro* inhibitory effects of different anthocyanins/anthocyanin-rich samples on carbohydrate digesting enzymes confirm inhibitory potential of anthocyanins towards  $\alpha$ -amylase and  $\alpha$ -glucosidase (Table 4.3) which may vary depending on their aglycone (sugar free part of anthocyanins) structure or sugar moieties.

*In vivo* data indicated that blackcurrant anthocyanins reduced the postprandial glycaemia *in vivo* at a dose of 600 mg (Castro-Acosta et al., 2016). In contrast, administration of higher dose blueberry anthocyanins (724 mg) has no significant effects on postprandial blood glucose levels (Bell et al., 2017). Regulation of postprandial hyperglycaemia is paramount in the management of diabetes and its complications (Sudhir & Mohan, 2002) that can be achieved by inhibiting the activity of carbohydrate metabolizing enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase, the key enzymes that convert complex carbohydrates into absorbable monosaccharides (Turina et al., 2006; Zhang et al., 2017). Polyphenolic compounds have shown glucoregulatory properties by delaying starch digestion, in particular, anthocyanin-rich berries such as raspberries, strawberries, blueberries and black currant have demonstrated inhibitory properties towards  $\alpha$ -amylase and  $\alpha$ -glucosidase *in vitro* (Tadera et al., 2006; Zhang et al., 2010; Sui et al., 2016) which has been confirmed in present study (Chapter 4). Recently, it has been demonstrated that extracts from anthocyanin-rich berry fruits are stronger inhibitors of  $\alpha$ -glucosidase than  $\alpha$ -amylase (Berger et al., 2020). The present *in vitro* data have also shown that blueberry juice is a stronger inhibitor of  $\alpha$ -glucosidase than the  $\alpha$ -amylase. These findings are in line with the literature demonstrating that blueberry extract reduced activity of  $\alpha$ -glucosidase ( $IC_{50}$  18  $\mu$ g/mL) more effectively than PPA ( $IC_{50}$  300  $\mu$ g/mL) (McDougall et al., 2005). Recently, Podsedek et al. (2014) has also reported  $\alpha$ -amylase ( $IC_{50}$  46.40 mg/mL) and  $\alpha$ -glucosidase (68.18 mg/mL) inhibitory activities of blueberry. Our *in vitro* data support the theory that BJ in the present study may have exerted inhibitory effects on the digestive enzymes,  $\alpha$ -amylase and  $\alpha$ -glucosidase *in vivo*, resulted in immediate postprandial effects in

humans. In the present study, almost 60% of the available carbohydrates were based on monosaccharides (glucose and fructose). It is likely that, in addition to inhibition of both enzymes, there might have also been inhibition of glucose uptake (Alzaid et al., 2013; Schulze et al., 2014), along with perhaps other mechanisms contributing to the strong effect on glucose response.

Different anthocyanin-rich berry extracts such as lingonberry, elderberry, bilberry and chokeberry have shown to enhance glucose uptake in HepG2-Cells (human liver cells) at concentration of 50 µg/mL (Ho et al., 2017). Cyanidin-3-O-β-glucoside and its metabolite protocatechuic acid exert insulin-like effects by activating PPAR $\gamma$ , increasing adiponectin secretion and enhancing GLUT4 expression in human omental adipocytes (Scazzocchio et al., 2011). Blueberry consumption has been reported to improve insulin sensitivity in non-diabetic insulin resistance men and women (Stull et al., 2010). Recently, increased insulin action was observed in T2DM patients who consumed 80 mg anthocyanins (purified from bilberry and blackcurrant) twice a day for 24 weeks (Alnajjar et al., 2020). In the current study, it is likely that reduced carbohydrate digestion through the inhibition of digestive enzyme has contributed to lower the postprandial glucose response *in vivo*. However, the contribution of other potential mechanisms needs to be investigated further to confirm the antidiabetic properties of blueberry.

A huge diversity in the structural properties of anthocyanins influences their stability (Adisakwattana et al., 2009; Akkarachiyasit et al., 2010; Castro-Acosta et al., 2017), solubility, and bonding ability with the digestive enzymes (Sui et al., 2016; Berger et al., 2020). Depending on their anthocyanins profile, the anthocyanin-rich extracts exert differ in their magnitude of inhibitory effects towards  $\alpha$ -amylase and  $\alpha$ -glucosidase *in vitro* (Chapter 4). It has been found that cyanidin-3-rutinoside (IC<sub>50</sub> 0.25 mM) inhibited the  $\alpha$ -glucosidase activity more effectively (Adisakwattana et al., 2011) than cyanidin-3-galactoside (0.5 mM), cyanidin-3-glucoside (IC<sub>50</sub> 0.97 mM) and cyanidin-3,5-diglucoside (IC<sub>50</sub> >2 mM) (Akkarachiyasit et al., 2010). Whereas cyanidin-3-glucoside was the most effective inhibitor of  $\alpha$ -amylase followed

by cyanidin-3-rutinoside, cyanidin 3,5-glucoside, and peonidin-3-glucoside with IC50 values of 0.024, 0.031, 0.04 and 0.075 mM, respectively (Sui et al., 2016).

Strengths of the present study include the consumption of test drinks with a starchy food (white bread) and sugar contents of all the drinks were adjusted to provide similar amount of available carbohydrate (50 g). Postprandial glucose response was reduced with both doses of blueberry compared to control but did not differ significantly between the two doses. The lower dose of blueberry juice used is achievable in everyday diets, as intake of 327 mg of blueberry anthocyanins is equivalent to approximately 55.5 g of fresh blueberries. Participants followed a low polyphenol diet for 24 h and fasted 8-10 h prior to each visit to minimize the influence of habitual polyphenol consumption.

## **5.7 Limitations of study**

Small sample size, lack of information on individual polyphenols/anthocyanins and their bioavailability are some of the limitations of the current study. Furthermore, insulin level (as an indicator to detect insulin resistance) was not measured in the current study. It has been demonstrated that plasma insulin concentrations follow the glucose response (Castro-Acosta et al., 2017). The results of HS glycaemic study (Chapter 3) confirmed these findings. In addition, the pH effect of the BJ was not considered in the present study which may contribute towards lowering postprandial glycaemic response.

## **5.8 Conclusion**

To conclude, this study confirmed that blueberry consumption reduced the postprandial glycaemia in humans. The bioactive ingredients of blueberry suppressed the activity of starch digesting enzymes which is an obvious mechanism observed for this hypoglycaemic effect. The effects of lower doses on postprandial glucose response should be investigated in future studies. Intake of blueberry should be encouraged to improve postprandial glycaemia, although the efficacy of blueberry from different types of food should be explored in future

research. Future studies assessing blueberry's effects on insulin and other mechanisms will be valuable for a better understanding of role of blueberries in glucose regulation/metabolism.

## Chapter 6

### General discussion

The worldwide burden of diabetes is increasing rapidly, and among the main causes of this increase are sedentary lifestyle, unhealthy dietary habits, aging, and being overweight. Regulation of postprandial hyperglycaemia and insulinaemia are among the key factors in the prevention and management of health conditions such as obesity, T2DM and cardiovascular disease (Blaak et al., 2012). Based on evidence from studies involving type 1 (Diabetes Control and Complication Trail; DCCT) and T2DM diabetic patients (UK Prospective Diabetes Study; UKPDS), it was suggested that controlling blood glucose to nearly target levels (normoglycaemic) not only reduced the incidence and progression of microvascular complication but also decreased the morbidity and mortality in patients with diabetes (DDTC, 1993; UKPDS, 1998). Therefore, it is imperative to control carbohydrate digestion and glucose absorption after high-carbohydrate meals.

Diet has long been linked with the progression and development of T2DM. Therefore, dietary changes are important to prevent T2DM, control existing diabetes and prevent or/and delay the rate of development of diabetic complications. An analysis of acute RCTs (n = 13) assessed the effects of polyphenol-rich sources consumed with starchy meals on postprandial glycaemia and indicated that polyphenols have potential to modulate the carbohydrate digestion and reduce the risk of T2DM (Coe & Ryan, 2016). Anthocyanins have been described number of biological activities such as anticancer, antimicrobial, antiobesity, antidiabetic and cardioprotective properties as indicated by many *in vitro* as well as *in vivo* studies (Yousuf et al., 2016; Li et al., 2017). In recent years, there has been an increasing interest in understanding the mechanisms related to antidiabetic effects of anthocyanins, in light of an inverse association between intake of anthocyanin-rich foods and the risk of T2DM suggested by numerous epidemiological studies (Seeram, 2012; Mursu et al., 2014; Joseph et al., 2016). This thesis on the antidiabetic of anthocyanins on postprandial hyperglycaemic has

covered three key areas in order to investigate the role of anthocyanins in the prevention and management of T2DM *in vivo* and *in vitro* and confirming their antidiabetic properties.

In the first instance (Chapter 5), postprandial effects of anthocyanin-rich blueberry drink were investigated *in vivo* to establish glycaemic response study which confirmed the glucose lowering properties of anthocyanins. Further, the dose dependent effects of anthocyanins containing HS drink on postprandial glucose and insulin (*in vivo*) were determined and compared to blueberry (Chapter 3). The contribution of individual anthocyanins/anthocyanins containing samples towards carbohydrate digesting enzymes were measured (*in vitro*) to determine the possible mechanisms by which anthocyanins and may exert beneficial effects on glycaemic markers (Chapter 4). *In vitro* methods to measure  $\alpha$ -amylase inhibition by pigmented samples such as anthocyanins or anthocyanin-rich extracts were compared in Chapter 4.

## **6.1 *In vivo* postprandial effects of *Hibiscus sabdariffa***

The overall aim of this research was to test the hypothesis that anthocyanins have the potential to affect postprandial glycaemic response *in vivo* and to inhibit the activity of carbohydrate digesting enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase) *in vitro*. The potential of anthocyanins to have a lowering effect on glucose and insulin after consumption of carbohydrate-rich meal with anthocyanin-rich drinks were investigated in healthy volunteers. The literature review (Chapter 1) shows different antidiabetic effects of anthocyanins in managing and preventing the T2DM. Inhibition of carbohydrate digesting enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase has been considered the most promising mechanism by which anthocyanins may reduce the postprandial hyperglycaemia, a major risk factor for T2DM. *In vivo* glycaemic investigations involving anthocyanin-rich foods have shown mixed outcomes with some studies have reducing effects on postprandial glycaemic response and a few showing an effect as discussed in Chapter 3 and 5. Several reasons such as different study designs anthocyanin source/dose, food matrix and control sample (whether bread, glucose or sucrose) may affect the results of these studies.

In this research, human intervention studies were designed to investigate the dose and source effects of anthocyanins on postprandial glycaemic response. An *in vivo* glycaemic study was conducted using blueberry, a more common and promising source of anthocyanins (Chapter 5). The most important objective was to establish whether anthocyanins have an effect on glycaemic response *in vivo*. Two different doses of blueberry were administered to determine whether the inhibition was dose dependent as is usually the case *in vitro*. In the second intervention, *Hibiscus sabdariffa* was used as an anthocyanin source (Chapter 3).

*Hibiscus sabdariffa* is rich in anthocyanins ranging from 172.6 mg/100 g to 4408 mg/100g (Wong et al., 2002; Chumsri et al., 2008; Abou-Arab et al., 2011; Borrás-Linares et al., 2015). HS has been well known for its blood pressure lowering effects as indicated by several studies (Mojiminiyi et al., 2007; Wahabi et al., 2010; Mardiah et al., 2014; Nwachukwu et al., 2015). It has been reported that the most abundant anthocyanins in HS, delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside (Sindi et al., 2014; Ifie et al., 2016) inhibited the activity of ACE (Angiotensin-converting enzyme) and possess antihypertensive effects (Ojeda et al., 2010). In addition, HS extracts have been reported beneficial to manage hyperlipidaemia and inhibiting atherosclerosis and coronary diseases due to its lipid lowering potential (Chang et al., 2006; Hirunpanich et al., 2006; Farombi & Ige, 2007; Yang et al., 2010). Some *in vitro* studies demonstrated the antidiabetic effects of HS (Hansawasdi et al., 2000; Ifie et al., 2016), however, the results are conflicting due to difference in detection methods and source of enzyme and substrate. Importantly, *in vivo* human studies investigating the beneficial effects of HS on glycaemic response are lacking/limited. Recently, an acute study conducted by Abubakar et al. (2019) demonstrated the cardioprotective role of HS consumption, however, no significant change in blood glucose and insulin were determined. However, this study was not specifically designed to determine glycaemic markers. The current HS glycaemic study was conducted to investigate the dose dependent effects of acute consumption of anthocyanin containing HS drink on postprandial glucose and insulin (*in vivo*)

The commercially available HS concentrate used in the present research has been analysed for its total anthocyanin, total polyphenol and total sugar contents (Chapter 3). Healthy subjects were recruited in this research to avoid any risk effects such as recent systematic review and meta-analysis has confirmed the hypotensive effects of HS (Ellis et al., 2021). Given that the  $\alpha$ -amylases hydrolyse starch into disaccharides, oligosaccharides and  $\alpha$ -limit dextrin after which  $\alpha$ -glucosidases hydrolyse the products of  $\alpha$ -amylase catalysed hydrolysis into glucose, bread combined with sucrose (as a substrate for glucosidase) was used as a carbohydrate source to test the inhibition of both  $\alpha$ -amylase and  $\alpha$ -glucosidase.

Previously, anthocyanin doses in the range of 300-600 mg have demonstrated reduction of postprandial glucose response (Torronen et al., 2010; Castro-Acosta et al., 2016). In the current blueberry study (Chapter 5), the two doses of BJ providing 327 (D1) and 545 mg (D2) anthocyanins were administered together with a carbohydrate source (bread+glucose+fructose). The results indicated significantly reduced glucose iAUC by D1 (22%) and D2 (20%) compared to the sugar-matched control group, irrespective of the dose applied. Dose-response effects on glycaemic response were observed in the acute study using HS, with the higher dose (220 mg) resulting in 18% reduction compared to low dose (132 mg) and 7% reduction, compared to control group. These data highlight that less than 300 mg of anthocyanins might be sufficient to reduce glycaemic response, which, in case of blueberries, requires further research to determine the minimum dose of anthocyanins that might be required for maximum postprandial glucose reduction. Since the anthocyanin patterns of both of these sources are different, as well as the presence of other compounds which may contribute to hypoglycaemic effects, further research is needed to delineate contribution of individual anthocyanins and other components on glycaemic response. In addition to bioactive effects, the pH of the product/drink needs to be taken into account that may contribute to postprandial effects as detailed in Chapter 3.

The human study data indicated blood glucose and insulin were significantly reduced in healthy subjects of different ethnicity following acute consumption of higher dose of HS

anthocyanins. The single serving of 50 mL HS concentrate that reduce the postprandial glucose and insulin response in the current study provides the similar benefits as 7 normal cups of HS tea.

## **6.2 *In vitro* carbohydrate inhibition**

The aim was to determine the mechanism responsible for the hypoglycaemic effects of HS. Several studies have reported that anthocyanins have potential to inhibit the activity of carbohydrate digesting enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase (Adisakwattana et al., 2009; Adisakwattana et al., 2011; Akkarachiyasit et al., 2011; Barik et al., 2020) and reduce the postprandial hyperglycaemia (Torrönen et al., 2010; Castro-Acosta et al., 2016). The activity of HS and its main anthocyanins and some of their metabolites (chlorogenic acid, gallic acid, protocatechuic acid) against  $\alpha$ -amylase and  $\alpha$ -glucosidase were determined.

The *in vitro* methods used for measuring antidiabetic properties of anthocyanins are lacking consistency in terms of reporting % inhibition, mode of inhibition, use of substrate and enzyme source and specific group of polyphenolic compounds responsible as inhibitors and their synergistic effects (Prpa et al., 2021). The most common method of detection used for  $\alpha$ -amylase and  $\alpha$ -glucosidase assays are colorimetric determination at 540 nm (for 3,5-dinitrosalicylic acid (DNS) method) and at 405 nm (for pNPG substrate), respectively. The glucosidase assay can be effected by the absorbance and/or intrinsic colour of anthocyanins which cause background signal and compromised the efficiency of this method. In the present work, performing the kinetic measurement and recording of product scan (Chapter 3) to determine the  $\alpha$ -glucosidase inhibitory activity of anthocyanin-rich extracts has provided with a better approach to overcome the colour interference/absorbance signal. The DNS method used to determine  $\alpha$ -amylase inhibition has some limitations including low sample throughput, many steps involved, potential interference of polyphenols with DNS reagent (unless SPE is used to remove these), which is discussed in detail in Chapter 4. Hence, the performance of an alternative method, utilizing a synthetic substrate CNPG3 with monitoring of absorbance change (indicating reaction product formation) was tested and compared with the results of

the DNS assay to determine the  $\alpha$ -amylase inhibitory potential of anthocyanins/anthocyanin-rich samples (Chapter 4). The present data indicate that enzyme inhibitory properties of pure anthocyanins (cyanidin and cyanidin galactopyranoside) and anthocyanin-rich samples (blackcurrant, blueberry, cherry, hibiscus and pomegranate) can be easily measured by using CNPG3 substrate without any additional step. The results (Table 4.3) demonstrated that direct chromogenic assay is more sensitive to measure enzyme inhibitory properties compared to DNS assay. Overall, anthocyanins/anthocyanin-rich extracts showed lower  $\alpha$ -amylase inhibition in both assays except for black currant and pomegranate. These findings are consistent with previous research (McDougall et al., 2005; Berger et al., 2020) indicating poor inhibition  $\alpha$ -amylase by individual anthocyanins or anthocyanins containing samples. The crude polyphenolic fractions (black carrot and mahaleb cheery) showed more inhibition than their purified anthocyanins fractions (Chapter 4).

The difference in enzyme inhibitory extent of different extracts could be explained by the polyphenolic/anthocyanin's composition of tested samples (Chapter 4). From all the fruit extracts analysed in this research, pomegranate had the highest polyphenol and lowest anthocyanin contents but exhibit strongest inhibition towards  $\alpha$ -amylase ( $IC_{50}$   $11.33 \pm 2.3$   $\mu\text{g/mL}$ ) and  $\alpha$ -glucosidase ( $IC_{50}$   $0.1 \pm 2.3$   $\mu\text{g/mL}$ ). These findings indicate that not only anthocyanins, but other polyphenolic compounds present in fruit extracts contribute towards carbohydrate inhibition. The results of the present *in vitro* HS study (Chapter 3) also confirmed the synergy of different components towards  $\alpha$ -glucosidase inhibition as pure compound were failed to demonstrate any inhibition or showed very less inhibition. *In vitro* enzyme inhibition data were in agreement with the results from the literature which show that different anthocyanins have different enzyme inhibitory properties (Kalita et al., 2018; Berger et al., 2020). The lower  $IC_{50}$  values of tested samples in case of direct chromogenic assay compared to DNS assay (Chapter 4) indicated the increased sensitivity of this method which was linked to short chain substrate. A potential disadvantage of using CNPG3 as a substrate is that this

is not a physiological substrate, however, it allows screening large number of samples in short time and provides basis for *in vivo* studies.

The mode of inhibition of  $\alpha$ -amylase by pure anthocyanins and purified anthocyanin-rich black currant extract was investigated using CNPG3 substrate and enzymatic kinetic parameters were determined (in the presence and absence of pure inhibitors). Kinetic parameters  $V_{max}$  and  $k_m$  obtained from Lineweaver-Burk plots indicate competitive and mixed-type inhibition by pure anthocyanins and blackcurrant, respectively. The results are in line with literature indicating competitive/non-competitive inhibitory effects of anthocyanins/anthocyanin-rich extracts against  $\alpha$ -amylase (Homoki et al., 2016; Sui et al., 2016; Ji et al., 2021). The present work highlighted the limitations of DNS assay as a standard *in vitro* method used for assessing the antidiabetic properties of pigmented samples and suggested the use of the direct chromogenic assay which is more affordable and time saving for screening large number of samples.

To determine the potential mechanism contributing towards postprandial effects *in vivo*, the HS and BJ concentrates were analysed for *in vitro* enzyme inhibitory properties towards  $\alpha$ -amylase and  $\alpha$ -glucosidase. The results indicated dose dependent inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase by blueberry juice, however, the inhibitory effects are more prominent in case of  $\alpha$ -glucosidase as also reported by McDougall et al. (2005). The hibiscus data demonstrated dose dependent inhibition of  $\alpha$ -glucosidase upon incubation with different doses of HS concentrate.

### **6.3 Impact and implication of this research**

The work from this PhD project investigated the glucoregulatory effects of anthocyanins and anthocyanin-rich extracts and possible mechanism through which anthocyanins may modulate the digestion of carbohydrates. Furthermore, *in vitro* methods to measure the antidiabetic properties of pigmented samples were compared. The *in vivo* investigations regarding the beneficial effects of *Hibiscus sabdariffa* in reducing the risk factors associated with diabetes

in this study were conducted under healthy conditions. The results demonstrate the potential of HS drink to lower blood glucose levels, and therefore, its consumption should be encouraged. In addition, HS should also be considered for functional food development, since it has demonstrated effective blood pressure lowering properties (Ellis et al., 2021). HS inhibited the activity of  $\alpha$ -glucosidase activity (*in vitro*), however, none of the individual anthocyanins from HS as well as anthocyanin metabolites tested, demonstrated enzyme inhibitory properties, indicating the hypoglycaemic effects might likely be a synergistic effect of different polyphenolic compounds present in HS. Given that the enhanced  $\alpha$ -glucosidase inhibition by HS combined with acarbose, utilization of HS into functional supplements may be of great interest as it will not only reduce the side effects associated with synthetic inhibitors used to manage diabetes, but also provide natural sources as enzyme inhibitors. This is the first study that is demonstrating hypoglycaemic effects of hibiscus in humans and potential mechanisms that are involved.

Globally, HS is known by different names, including karkad'e in Arabic regions, Roselle, or red/sour tea in the UK. In Thailand, it is called karchiap daeng, and in Spanish, Jamaica. It is famous as l'oiselle in France. The fact that HS is consumed (as a cold/hot drink) in different parts of the world, HS is at present mainly added to tea mixtures as a colouring agent. The current study demonstrating the beneficial effects of HS on postprandial response is thus providing evidence on the importance of HS for the metabolic health and prevention of chronic disease. There is huge potential for future research in developing functional/novel product based on HS.

Hyperglycaemia refers to a rise in blood sugar levels, a defining characteristic of T2DM and its management/treatment. Antidiabetic medications such as acarbose, voglibose and miglitol slow down the carbohydrate digestion via the inhibition of digestive enzymes and reduce the postprandial glucose excursion (Derosa & Maffioli, 2012). In recent years, polyphenolic compounds, in particular, anthocyanins gained much popularity due to their role in the management and prevention of T2DM. The present *in vitro* data determined more inhibition of

$\alpha$ -glucosidase compared to  $\alpha$ -amylase by all the samples (pure anthocyanins/anthocyanin-rich extracts/crude/purified anthocyanins fractions). Due to the presence of other polyphenolic compounds, anthocyanins alone cannot account for the bioactivity of drinks rich in anthocyanins (Berger et al., 2020). In addition, higher  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition was determined for the polyphenolic fractions compared to purified anthocyanin fractions (Chapter 4). Given that pure HS anthocyanins failed to demonstrate inhibitory effects on  $\alpha$ -amylase and  $\alpha$ -glucosidase, further research is needed to determine whether different polyphenolic compounds contribute synergistically to enzymatic inhibitory properties of HS.

There is scientific evidence that different polyphenol levels can influence the magnitude of health outcomes (Blumberg et al., 2015; Castro-Acosta et al., 2017). The present research has shown that hypoglycaemic effects of anthocyanins were dose dependent. Overall, *in vivo* studies demonstrated that commercially available pure concentrated juice of hibiscus and blueberry (with no added sugar) delayed the appearance of glucose in the blood and inhibited the activity of digestive enzymes. Although, consumption of hibiscus juice/drink is less common, it can be combined with other anthocyanin-rich drinks to get optimal benefits.

Taken together, the present work will have strong potential to impact the field of anthocyanins and health research, in providing insight into mechanisms of action and *in vivo* evidence of the acute effects of anthocyanins-rich foods in preventing and managing T2DM, highlighting the importance of examining effect of anthocyanin containing foods on specific subject groups as well as establishing *in vitro* and *in vivo* methods to evaluate anthocyanins for their potential in improving human health.

## **6.4 Conclusion and future perspectives**

Overall, this research has shown that anthocyanin-rich drinks (hibiscus and blueberry) significantly reduced the postprandial glucose response to carbohydrate-rich foods (white bread) in healthy volunteers by inhibiting the activity of carbohydrate digestive enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase). To best of our knowledge, this is the first human study

demonstrated hypoglycaemic effects of *Hibiscus sabdariffa*. Since anthocyanin-rich drinks/foods reduced the postprandial glycaemic response in healthy subjects, future interventions studies investigating glucoregulatory effects of HS in diabetic and pre-diabetic subjects are warranted. In addition, chronic trials are needed to establish the long-term effects of anthocyanin-rich interventions on glycaemic markers, including fasting blood glucose (FBG), fasting blood insulin (FBI), formation of advanced glycation end products and homeostatic model assessment-insulin resistance (HOMA-IR). Since blueberry demonstrated stronger postprandial effects than HS, the present study identified that the magnitude of hypoglycaemic effects differed depending on the anthocyanin source. The *in vivo* data were in with *in vitro* findings indicated different inhibitory potential of different anthocyanins/anthocyanin containing samples towards  $\alpha$ -amylase and  $\alpha$ -glucosidase.

Given that individual HS compounds have not shown any inhibitory effects towards  $\alpha$ -amylase and  $\alpha$ -glucosidase, anthocyanins alone cannot account for the bioactivity of HS. Therefore, further studies are envisaged to determine the contribution of other polyphenols and their interactions towards reduced carbohydrate digestion. The lower pH (>3) of the intervention could also affect the glycaemic profile by attenuating the salivary amylase activity which, although observed for HS in the current work, requires further investigations to better understand the contribution of pH towards enzyme inhibition and other possible glucoregulatory mechanisms that may contribute to antihyperglycaemic and other metabolic disease related effects of anthocyanins.

## References

- Abou-Arab, A.A., Abu-Salem, F.M. and Abou-Arab, E.A. 2011. Physico-chemical properties of natural pigments (anthocyanin) extracted from Roselle calyces (*Hibiscus subdariffa*). *Journal of American Science*. **7**(7), pp.445-456.
- Abubakar, S.M., Ukeyima, M.T., Spencer, J.P.E. and Lovegrove, J.A. 2019. Acute Effects of Hibiscus Sabdariffa Calyces on Postprandial Blood Pressure, Vascular Function, Blood Lipids, Biomarkers of Insulin Resistance and Inflammation in Humans. *Nutrients*. **11**(2).
- Ademiluyi, A.O. and Oboh, G. 2013. Aqueous extracts of Roselle (*Hibiscus sabdariffa* Linn.) varieties inhibit alpha-amylase and alpha-glucosidase activities in vitro. *Journal of Medicinal Food*. **16**(1), pp.88-93.
- Adisakwattana, S., Charoenlertkul, P. and Yibchok-Anun, S. 2009. alpha-Glucosidase inhibitory activity of cyanidin-3-galactoside and synergistic effect with acarbose. *Journal of Enzyme Inhibition and Medicinal Chemistry*. **24**(1), pp.65-69.
- Adisakwattana, S., Ngamrojanavanich, N., Kalampakorn, K., Tiravanit, W., Roengsumran, S. and Yibchok-Anun, S. 2004. Inhibitory activity of cyanidin-3-rutinoside on alpha-glucosidase. *Journal of Enzyme Inhibition and Medicinal Chemistry*. **19**(4), pp.313-316.
- Adisakwattana, S., Ruengsamran, T., Kampa, P. and Sompong, W. 2012. In vitro inhibitory effects of plant-based foods and their combinations on intestinal  $\alpha$ -glucosidase and pancreatic  $\alpha$ -amylase. *BMC Complementary and Alternative Medicine*. **12**, p110.
- Adisakwattana, S., Yibchok-Anun, S., Charoenlertkul, P. and Wongsasiripat, N. 2011. Cyanidin-3-rutinoside alleviates postprandial hyperglycemia and its synergism with acarbose by inhibition of intestinal  $\alpha$ -glucosidase. *Journal of Clinical Biochemistry and Nutrition*. **49**(1), pp.36-41.
- Aguiar, L.M. and Cazarin, C.B.B. 2021. In vitro and in vivo methods to predict carbohydrate bioaccessibility. *Current Opinion in Food Science*. **42**, pp.69-75.
- Akkarachiyasit, S., Charoenlertkul, P., Yibchok-Anun, S. and Adisakwattana, S. 2010. Inhibitory activities of cyanidin and its glycosides and synergistic effect with acarbose against intestinal alpha-glucosidase and pancreatic alpha-amylase. *International Journal of Molecular Sciences*. **11**(9), pp.3387-3396.
- Akkarachiyasit, S., Yibchok-Anun, S., Wacharasindhu, S. and Adisakwattana, S. 2011. In vitro inhibitory effects of cyanidin-3-rutinoside on pancreatic alpha-amylase and its combined effect with acarbose. *Molecules*. **16**(3), pp.2075-2083.
- Al-Goblan, A.S., Al-Alfi, M.A. and Khan, M.Z. 2014. Mechanism linking diabetes mellitus and obesity. *Diabetes, metabolic syndrome and obesity : targets and therapy*. **7**, pp.587-591.
- Alegbe, E.O., Terali, K., Olofinsan, K.A., Surgun, S., Ogbaga, C.C. and Ajiboye, T.O. 2019. Antidiabetic activity-guided isolation of gallic and protocatechuic acids from *Hibiscus sabdariffa* calyces. *Journal of Food Biochemistry* **43**(7), pe12927.
- Ali, B.H., Mousa, H.M. and El-Mougy, S. 2003. The effect of a water extract and anthocyanins of hibiscus sabdariffa L on paracetamol-induced hepatotoxicity in rats. *Phytotherapy Research*. **17**(1), pp.56-59.
- Alnajjar, M., Kumar Barik, S., Bestwick, C., Campbell, F., Cruickshank, M., Farquharson, F., Holtrop, G., Horgan, G., Louis, P., Moar, K.-M., Russell, W.R., Scobbie, L. and Hoggard, N. 2020. Anthocyanin-enriched bilberry extract attenuates glycaemic response in overweight volunteers without changes in insulin. *Journal of Functional Foods*. **64**.

- Alzaid, F., Cheung, H.-M., Preedy, V.R. and Sharp, P.A. 2013. Regulation of Glucose Transporter Expression in Human Intestinal Caco-2 Cells following Exposure to an Anthocyanin-Rich Berry Extract. *PLoS One*. **8**(11), pe78932.
- American Diabetes, A. 2010. Diagnosis and classification of diabetes mellitus. *Diabetes Care*. **33** Suppl 1(Suppl 1), pp.S62-S69.
- Andersen, Ø.M. and Jordheim, M. 2010. Anthocyanins. *Encyclopedia of Life Sciences*.
- Andraini, T. and Yolanda, S. 2014. Prevention of insulin resistance with Hibiscus sabdariffa Linn. extract in high-fructose fed rat. *Medical Journal of Indonesia*. **23**(4), pp.192-196.
- Barik, S.K., Russell, W.R., Moar, K.M., Cruickshank, M., Scobbie, L., Duncan, G. and Hoggard, N. 2020. The anthocyanins in black currants regulate postprandial hyperglycaemia primarily by inhibiting alpha-glucosidase while other phenolics modulate salivary alpha-amylase, glucose uptake and sugar transporters. *Journal of Nutritional Biochemistry*. **78**, p108325.
- Bell, L., Lamport, D.J., Butler, L.T. and Williams, C.M. 2017. A study of glycaemic effects following acute anthocyanin-rich blueberry supplementation in healthy young adults. *Food and Function*. **8**(9), pp.3104-3110.
- Bellesia, A., Verzelloni, E. and Tagliacozzi, D. 2015. Pomegranate ellagitannins inhibit alpha-glucosidase activity in vitro and reduce starch digestibility under simulated gastro-intestinal conditions. *International Journal of Food Sciences and Nutrition*. **66**(1), pp.85-92.
- Berger, K., Ostberg-Potthoff, J.J., Bakuradze, T., Winterhalter, P. and Richling, E. 2020. Carbohydrate Hydrolase-Inhibitory Activity of Juice-Based Phenolic Extracts in Correlation to Their Anthocyanin/Copigment Profile. *Molecules*. **25**(22), p5224.
- Blaak, E.E., Antoine, J.M., Benton, D., Björck, I., Bozzetto, L., Brouns, F., Diamant, M., Dye, L., Hulshof, T., Holst, J.J., Lamport, D.J., Laville, M., Lawton, C.L., Meheust, A., Nilsson, A., Normand, S., Rivellese, A.A., Theis, S., Torekov, S.S. and Vinoy, S. 2012. Impact of postprandial glycaemia on health and prevention of disease. *Obesity reviews : an official journal of the International Association for the Study of Obesity*. **13**(10), pp.923-984.
- Blando, F., Calabriso, N., Berland, H., Maiorano, G., Gerardi, C., Carluccio, M.A. and Andersen, O.M. 2018. Radical Scavenging and Anti-Inflammatory Activities of Representative Anthocyanin Groupings from Pigment-Rich Fruits and Vegetables. *International Journal of Food Sciences and Nutrition*. **19**(1).
- Blumberg, J.B., Vita, J.A. and Chen, C.Y. 2015. Concord Grape Juice Polyphenols and Cardiovascular Risk Factors: Dose-Response Relationships. *Nutrients*. **7**(12), pp.10032-10052.
- Bordoni, A., Boesch, C., Malpuech-Brugere, C., Orfila, C. and Tomas-Cobos, L. 2019. The role of bioactives in energy metabolism and metabolic syndrome. *Proceedings of the Nutrition Society*. **78**(3), pp.340-350.
- Borrás-Linares, I., Fernández-Arroyo, S., Arráez-Roman, D., Palmeros-Suárez, P.A., Del Val-Díaz, R., Andrade-González, I., Fernández-Gutiérrez, A., Gómez-Leyva, J.F. and Segura-Carretero, A. 2015. Characterization of phenolic compounds, anthocyanidin, antioxidant and antimicrobial activity of 25 varieties of Mexican Roselle (*Hibiscus sabdariffa*). *Industrial Crops and Products*. **69**, pp.385-394.
- Briguglio, G., Costa, C., Pollicino, M., Giambo, F., Catania, S. and Fenga, C. 2020. Polyphenols in cancer prevention: New insights. *International Journal of Functional Nutrition*. **1**(2), pp.1-1.
- Brouns, F., Björck, I., Frayn, K.N., Gibbs, A.L., Lang, V., Slama, G. and Wolever, T.M.S. 2005. Glycaemic index methodology. *Nutrition Research Reviews*. **18**(1), pp.145-171.
- Bryant, N.J., Govers, R. and James, D.E. 2002. Regulated transport of the glucose transporter GLUT4. *Nature reviews Molecular cell biology*. **3**(4), pp.267-277.

- Bueno, J.M., Sáez-Plaza, P., Ramos-Escudero, F., Jiménez, A.M., Fett, R. and Asuero, A.G. 2012. Analysis and Antioxidant Capacity of Anthocyanin Pigments. Part II: Chemical Structure, Color, and Intake of Anthocyanins. *Critical Reviews in Analytical Chemistry*. **42**(2), pp.126-151.
- Bunea, A., Rugina, D., Sconta, Z., Pop, R.M., Pinteá, A., Socaciu, C., Tabaran, F., Grootaert, C., Struijs, K. and VanCamp, J. 2013. Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells. *Phytochemistry*. **95**, pp.436-444.
- Cao, G., Russell, R.M., Lischner, N. and Prior, R.L. 1998. Serum Antioxidant Capacity Is Increased by Consumption of Strawberries, Spinach, Red Wine or Vitamin C in Elderly Women. *The Journal of Nutrition*. **128**(12), pp.2383-2390.
- Carbohydrates in human nutrition. Report of a Joint FAO/WHO Expert Consultation. 1998. *FAO Food and Nutrition Paper*. **66**, pp.1-140.
- Carvajal-Zarrabal, O., Waliszewski, S.M., Barradas-Dermitz, D.M., Orta-Flores, Z., Hayward-Jones, P.M., Nolasco-Hipolito, C., Angulo-Guerrero, O., Sanchez-Ricano, R., Infanzon, R.M. and Trujillo, P.R. 2005. The consumption of Hibiscus sabdariffa dried calyx ethanolic extract reduced lipid profile in rats. *Plant Foods for Human Nutrition*. **60**(4), pp.153-159.
- Castro-Acosta, M.L., Smith, L., Miller, R.J., McCarthy, D.I., Farrimond, J.A. and Hall, W.L. 2016. Drinks containing anthocyanin-rich blackcurrant extract decrease postprandial blood glucose, insulin and incretin concentrations. *Journal of Nutritional Biochemistry*. **38**, pp.154-161.
- Castro-Acosta, M.L., Stone, S.G., Mok, J.E., Mhajan, R.K., Fu, C.I., Lenihan-Geels, G.N., Corpe, C.P. and Hall, W.L. 2017. Apple and blackcurrant polyphenol-rich drinks decrease postprandial glucose, insulin and incretin response to a high-carbohydrate meal in healthy men and women. *Journal of Nutritional Biochemistry*. **49**, pp.53-62.
- Chang, H.C., Peng, C.H., Yeh, D.M., Kao, E.S. and Wang, C.J. 2014. Hibiscus sabdariffa extract inhibits obesity and fat accumulation, and improves liver steatosis in humans. *Food and Function*. **5**(4), pp.734-739.
- Chang, Y.C., Huang, K.X., Huang, A.C., Ho, Y.C. and Wang, C.J. 2006. Hibiscus anthocyanins-rich extract inhibited LDL oxidation and oxLDL-mediated macrophages apoptosis. *Food and Chemical Toxicology* **44**(7), pp.1015-1023.
- Chaudhury, A., Duvoor, C., Reddy Dendi, V.S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N.S., Montales, M.T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, C.K., Lohani, G.P. and Mirza, W. 2017. Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management. *Frontiers in Endocrinology*. **8**, pp.6-6.
- Chen, C.-C., Hsu, J.-D., Wang, S.-F., Chiang, H.-C., Yang, M.-Y., Kao, E.-S., Ho, Y.-C. and Wang, C.-J. 2003. Hibiscus sabdariffa extract inhibits the development of atherosclerosis in cholesterol-fed rabbits. *Journal of agricultural and food chemistry*. **51**(18), pp.5472-5477.
- Chen, L., Tuo, B. and Dong, H. 2016. Regulation of Intestinal Glucose Absorption by Ion Channels and Transporters. *Nutrients*. **8**(1), p43.
- Chen, Z., Li, W., Guo, Q., Xu, L., Santhanam, R.K., Gao, X., Chen, Y., Wang, C., Panichayupakaranant, P. and Chen, H. 2019. Anthocyanins from dietary black soybean potentiate glucose uptake in L6 rat skeletal muscle cells via up-regulating phosphorylated Akt and GLUT4. *Journal of Functional Foods*. **52**, pp.663-669.
- Chumsri, P., Anchalee, S. and Itharat, A. 2008. Studies on the optimum conditions for the extraction and concentration of roselle (Hibiscus sabdariffa Linn.) extract. *Songklanakarín Journal of Science and Technology*. **30**, pp.133-139.

- Clegg, M.E., Pratt, M., Meade, C.M. and Henry, C.J. 2011. The addition of raspberries and blueberries to a starch-based food does not alter the glycaemic response. *British Journal of Nutrition*. **106**(3), pp.335-338.
- Coe, S. and Ryan, L. 2016. Impact of polyphenol-rich sources on acute postprandial glycaemia: a systematic review. *Journal of Nutritional Science*. **5**, pp.e24-e24.
- Coe, S. and Ryan, L. 2016. White bread enriched with polyphenol extracts shows no effect on glycemic response or satiety, yet may increase postprandial insulin economy in healthy participants. *Nutrition Research*. **36**(2), pp.193-200.
- Control, C.f.D. and Prevention. 2013. *Diabetes data and trends: number (in millions) of civilian, noninstitutionalized persons with diagnosed diabetes, United States, 1980–2011*.
- Da Silva Pinto, M., Kwon, Y.-I., Apostolidis, E., Lajolo, F.M., Genovese, M.I. and Shetty, K. 2010. Evaluation of Red Currants (*Ribes Rubrum* L.), Black Currants (*Ribes Nigrum* L.), Red and Green Gooseberries (*Ribes Uva-Crispa*) for Potential Management of Type 2 Diabetes and Hypertension Using in Vitro models. *Journal of Food Biochemistry*.
- Damager, I., Numao, S., Chen, H., Brayer, G.D. and Withers, S.G. 2004. Synthesis and characterisation of novel chromogenic substrates for human pancreatic alpha-amylase. *Carbohydrate Research*. **339**(10), pp.1727-1737.
- DDTC. 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England journal of medicine*. **329**(14), pp.977-986.
- de Pascual-Teresa, S. and Sanchez-Ballesta, M.T. 2007. Anthocyanins: from plant to health. *Phytochemistry Reviews*. **7**(2), pp.281-299.
- DeFuria, J., Bennett, G., Strissel, K.J., Perfield, J.W., 2nd, Milbury, P.E., Greenberg, A.S. and Obin, M.S. 2009. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *Journal of Nutrition*. **139**(8), pp.1510-1516.
- Derosa, G. and Maffioli, P. 2012.  $\alpha$ -Glucosidase inhibitors and their use in clinical practice. *Archives of medical science : AMS*. **8**(5), pp.899-906.
- Deshpande, A.D., Harris-Hayes, M. and Schootman, M. 2008. Epidemiology of Diabetes and Diabetes-Related Complications. *Physical Therapy*. **88**(11), pp.1254-1264.
- Diabetes Prevention Program, R.G. 2012. Long-term safety, tolerability, and weight loss associated with metformin in the Diabetes Prevention Program Outcomes Study. *Diabetes Care*. **35**(4), pp.731-737.
- Dimitriadis, G.D., Maratou, E., Kountouri, A., Board, M. and Lambadiari, V. 2021. Regulation of Postabsorptive and Postprandial Glucose Metabolism by Insulin-Dependent and Insulin-Independent Mechanisms: An Integrative Approach. *Nutrients*. **13**(1), p159.
- Dona, A.C., Pages, G., Gilbert, R.G. and Kuchel, P.W. 2010. Digestion of starch: In vivo and in vitro kinetic models used to characterise oligosaccharide or glucose release. *Carbohydrate Polymers*. **80**(3), pp.599-617.
- Dreux, M. and Lafosse, M. 1995. Chapter 13 Evaporative Light Scattering Detection of Carbohydrates in HPLC. In: El Rassi, Z. ed. *Journal of Chromatography Library*. Elsevier, pp.515-540.
- Drews, G., Krippeit-Drews, P. and Düfer, M. 2010. Oxidative stress and beta-cell dysfunction. *Pflügers Archiv - European Journal of Physiology*. **460**(4), pp.703-718.

- Edirisinghe, I., Banaszewski, K., Cappozzo, J., Sandhya, K., Ellis, C.L., Tadapaneni, R., Kappagoda, C.T. and Burton-Freeman, B.M. 2011. Strawberry anthocyanin and its association with postprandial inflammation and insulin. *British Journal of Nutrition*. **106**(6), pp.913-922.
- Eleazu, C.O. 2016. The concept of low glycemic index and glycemic load foods as panacea for type 2 diabetes mellitus; prospects, challenges and solutions. *African Health Sciences*. **16**(2), pp.468-479.
- Ellis, L.R., Zulfiqar, S., Holmes, M., Marshall, L., Dye, L. and Boesch, C. 2021. A systematic review and meta-analysis of the effects of Hibiscus sabdariffa on blood pressure and cardiometabolic markers. *Nutrition Reviews*.
- Farombi, E.O. and Ige, O.O. 2007. Hypolipidemic and antioxidant effects of ethanolic extract from dried calyx of Hibiscus sabdariffa in alloxan-induced diabetic rats. *Fundamental and Clinical Pharmacology*. **21**(6), pp.601-609.
- Farooque, S., Rose, P.M., Benohoud, M., Blackburn, R.S. and Rayner, C.M. 2018. Enhancing the Potential Exploitation of Food Waste: Extraction, Purification, and Characterization of Renewable Specialty Chemicals from Blackcurrants ( *Ribes nigrum* L.). *Journal of Agricultural and Food Chemistry*. **66**(46), pp.12265-12273.
- Federlin, K. 2012. *Immunopathology of insulin: clinical and experimental studies*. Springer Science & Business Media.
- Fernando, G.S.N., Wood, K., Papaioannou, E.H., Marshall, L.J., Sergeeva, N.N. and Boesch, C. 2021. Application of an Ultrasound-Assisted Extraction Method to Recover Betalains and Polyphenols from Red Beetroot Waste. *ACS Sustainable Chemistry & Engineering*.
- Frank, T., Netzel, G., Kammerer, D.R., Carle, R., Kler, A., Kriesl, E., Bitsch, I., Bitsch, R. and Netzel, M. 2012. Consumption of Hibiscus sabdariffa L. aqueous extract and its impact on systemic antioxidant potential in healthy subjects. *Journal of the Science of Food and Agriculture*. **92**(10), pp.2207-2218.
- Freitas, D., Boue, F., Benallaoua, M., Airinei, G., Benamouzig, R. and Le Feunteun, S. 2021. Lemon juice, but not tea, reduces the glycemic response to bread in healthy volunteers: a randomized crossover trial. *European Journal of Nutrition*. **60**(1), pp.113-122.
- Freitas, D. and Le Feunteun, S. 2018. Acid induced reduction of the glycaemic response to starch-rich foods: the salivary alpha-amylase inhibition hypothesis. *Food and Function*. **9**(10), pp.5096-5102.
- Freitas, D. and Le Feunteun, S. 2019. Oro-gastro-intestinal digestion of starch in white bread, wheat-based and gluten-free pasta: Unveiling the contribution of human salivary alpha-amylase. *Food Chemistry*. **274**, pp.566-573.
- Fu, Z., Gilbert, E.R. and Liu, D. 2013. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Current Diabetes Reviews*. **9**(1), pp.25-53.
- Gondokesumo, M.E., Kusuma, H.S.W. and Widowati, W. 2017.  $\alpha$ -/ $\beta$ -Glucosidase and  $\alpha$ -Amylase Inhibitory Activities of Roselle (*Hibiscus sabdariffa* L.) Ethanol Extract. *Molecular and Cellular Biomedical Sciences*. **1**(1).
- Graham, T.E., Yang, Q., Blüher, M., Hammarstedt, A., Ciaraldi, T.P., Henry, R.R., Wason, C.J., Oberbach, A., Jansson, P.A., Smith, U. and Kahn, B.B. 2006. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *New England Journal of Medicine*. **354**(24), pp.2552-2563.
- Gromova, L.V., Fetissov, S.O. and Gruzdkov, A.A. 2021. Mechanisms of Glucose Absorption in the Small Intestine in Health and Metabolic Diseases and Their Role in Appetite Regulation. *Nutrients*. **13**(7), p2474.
- Grussu, D., Stewart, D. and McDougall, G.J. 2011. Berry polyphenols inhibit  $\alpha$ -amylase in vitro: identifying active components in rowanberry and raspberry. *Journal of Agricultural and Food Chemistry*. **59**(6), pp.2324-2331.

- Gurrola-Diaz, C.M., Garcia-Lopez, P.M., Sanchez-Enriquez, S., Troyo-Sanroman, R., Andrade-Gonzalez, I. and Gomez-Leyva, J.F. 2010. Effects of Hibiscus sabdariffa extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy). *Phytomedicine*. **17**(7), pp.500-505.
- Gutierrez-Albanchez, E., Kirakosyan, A., Bolling, S.F., García-Villaraco, A., Gutierrez-Mañero, J. and Ramos-Solano, B. 2019. Biotic elicitation as a tool to improve strawberry and raspberry extract potential on metabolic syndrome-related enzymes in vitro. *Journal of the Science of Food and Agriculture*. **99**(6), pp.2939-2946.
- Haji Faraji, M. and Haji Tarkhani, A. 1999. The effect of sour tea (Hibiscus sabdariffa) on essential hypertension. *Journal of Ethnopharmacology*. **65**(3), pp.231-236.
- Hajifaraji, M., Matlabi, M., Ahmadzadeh-Sani, F., Mehrabi, Y., Rezaee, M.S., Hajimehdipour, H., Hasanzadeh, A. and Roghani, K. 2018. Effects of aqueous extracts of dried calyx of sour tea (Hibiscus sabdariffa L.) on polygenic dyslipidemia: A randomized clinical trial. *Avicenna journal of phytomedicine*. **8**(1), pp.24-32.
- Hamza, A.A., Saleh Ksiksi, T. and Ali A Shamsi, O. 2015.  $\alpha$ -Glucosidase Inhibitory Activity of Common Traditional Medicinal Plants Used for Diabetes Mellitus. *Journal of Developing Drugs*. **04**(05).
- Hanamura, T., Mayama, C., Aoki, H., Hirayama, Y. and Shimizu, M. 2006. Antihyperglycemic effect of polyphenols from Acerola (Malpighia emarginata DC.) fruit. *Bioscience, Biotechnology, and Biochemistry*. **70**(8), pp.1813-1820.
- Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H. and Poutanen, K. 2010. Impact of dietary polyphenols on carbohydrate metabolism. *International Journal of Molecular Sciences*. **11**(4), pp.1365-1402.
- Hansawasdi, C., Kawabata, J. and Kasai, T. 2000. Alpha-amylase inhibitors from roselle (Hibiscus sabdariffa Linn.) tea. *Bioscience, Biotechnology, and Biochemistry*. **64**(5), pp.1041-1043.
- Hätönen, K.A., Similä, M.E., Virtamo, J.R., Eriksson, J.G., Hannila, M.-L., Sinkko, H.K., Sundvall, J.E., Mykkänen, H.M. and Valsta, L.M. 2006. Methodologic considerations in the measurement of glycemic index: glycemic response to rye bread, oatmeal porridge, and mashed potato. *The American Journal of Clinical Nutrition*. **84**(5), pp.1055-1061.
- Herrera-Arellano, A., Flores-Romero, S., Chavez-Soto, M. and Tortoriello, J. 2004. Effectiveness and tolerability of a standardized extract from Hibiscus sabdariffa in patients with mild to moderate hypertension: a controlled and randomized clinical trial. *Phytomedicine*. **11**(5), pp.375-382.
- Herrera-Arellano, A., Miranda-Sánchez, J., Avila-Castro, P., Herrera-Alvarez, S., Jiménez-Ferrer, J.E., Zamilpa, A., Román-Ramos, R., Ponce-Monter, H. and Tortoriello, J. 2007. Clinical effects produced by a standardized herbal medicinal product of Hibiscus sabdariffa on patients with hypertension. A randomized, double-blind, lisinopril-controlled clinical trial. *Planta Medica*. **73**(1), pp.6-12.
- Hidalgo, J., Flores, C., Hidalgo, M.A., Perez, M., Yañez, A., Quiñones, L., Caceres, D.D. and Burgos, R.A. 2014. Delphinol® standardized maqui berry extract reduces postprandial blood glucose increase in individuals with impaired glucose regulation by novel mechanism of sodium glucose cotransporter inhibition. *Panminerva Medica*. **56**(2 Suppl 3), pp.1-7.
- Hirunpanich, V., Utaipat, A., Morales, N.P., Bunyaphatsara, N., Sato, H., Herunsale, A. and Suthisisang, C. 2006. Hypocholesterolemic and antioxidant effects of aqueous extracts from the dried calyx of Hibiscus sabdariffa L. in hypercholesterolemic rats. *Journal of Ethnopharmacology*. **103**(2), pp.252-260.
- Ho, G.T.T., Nguyen, T.K.Y., Kase, E.T., Tadesse, M., Barsett, H. and Wangensteen, H. 2017. Enhanced Glucose Uptake in Human Liver Cells and Inhibition of Carbohydrate Hydrolyzing Enzymes by Nordic Berry Extracts. *Molecules*. **22**(10).

- Homoki, J.R., Nemes, A., Fazekas, E., Gyemant, G., Balogh, P., Gal, F., Al-Asri, J., Mortier, J., Wolber, G., Babinszky, L. and Remenyik, J. 2016. Anthocyanin composition, antioxidant efficiency, and alpha-amylase inhibitor activity of different Hungarian sour cherry varieties (*Prunus cerasus* L.). *Food Chemistry*. **194**, pp.222-229.
- Hong, S.H., Heo, J.-I., Kim, J.-H., Kwon, S.-O., Yeo, K.-M., Bakowska-Barczak, A.M., Kolodziejczyk, P., Ryu, O.-H., Choi, M.-K. and Kang, Y.-H. 2013. Antidiabetic and Beta cell-protection activities of purple corn anthocyanins. *Biomolecules & Therapeutics*. **21**(4), p284.
- Hopkins, A.L., Lamm, M.G., Funk, J.L. and Ritenbaugh, C. 2013. Hibiscus sabdariffa L. in the treatment of hypertension and hyperlipidemia: a comprehensive review of animal and human studies. *Fitoterapia*. **85**, pp.84-94.
- Howard, L.R., Brownmiller, C., Mauromoustakos, A. and Prior, R.L. 2016. Improved stability of blueberry juice anthocyanins by acidification and refrigeration. *Journal of Berry Research*. **6**, pp.189-201.
- Howard, L.R., Prior, R.L., Liyanage, R. and Lay, J.O. 2012. Processing and Storage Effect on Berry Polyphenols: Challenges and Implications for Bioactive Properties. *Journal of Agricultural and Food Chemistry*. **60**(27), pp.6678-6693.
- Huang, T.W., Chang, C.L., Kao, E.S. and Lin, J.H. 2015. Effect of Hibiscus sabdariffa extract on high fat diet-induced obesity and liver damage in hamsters. *Food & Nutrition Research*. **59**, p29018.
- Ifie, I., Marshall, L.J., Ho, P. and Williamson, G. 2016. Hibiscus sabdariffa (Roselle) Extracts and Wine: Phytochemical Profile, Physicochemical Properties, and Carbohydrase Inhibition. *Journal of Agricultural and Food Chemistry*. **64**(24), pp.4921-4931.
- Inaguma, T., Han, J. and Isoda, H. 2011. Improvement of insulin resistance by Cyanidin 3-glucoside, anthocyanin from black beans through the up-regulation of GLUT4 gene expression. *BMC Proceedings*. **5 Suppl 8**(Suppl 8), pp.P21-P21.
- Istek, N. and Gurbuz, O. 2017. Investigation of the impact of blueberries on metabolic factors influencing health. *Journal of Functional Foods*. **38**, pp.298-307.
- Jayaprakasam, B., Olson, L.K., Schutzki, R.E., Tai, M.H. and Nair, M.G. 2006. Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (*Cornus mas*). *Journal of Agricultural and Food Chemistry*. **54**(1), pp.243-248.
- Jenkins, D.J., Kendall, C.W., Augustin, L.S., Franceschi, S., Hamidi, M., Marchie, A., Jenkins, A.L. and Axelsen, M. 2002. Glycemic index: overview of implications in health and disease. *The American Journal of Clinical Nutrition*. **76**(1), pp.266S-273S.
- Jennings, A., Welch, A.A., Spector, T., Macgregor, A. and Cassidy, A. 2014. Intakes of anthocyanins and flavones are associated with biomarkers of insulin resistance and inflammation in women. *Journal of Nutrition*. **144**(2), pp.202-208.
- Jéquier, E. 1994. Carbohydrates as a source of energy. *The American Journal of Clinical Nutrition*. **59**(3), pp.682S-685S.
- Ji, Y., Liu, D., jin, Y., Zhao, J., Zhao, J., Li, H., Li, L., Zhang, H. and Wang, H. 2021. In vitro and in vivo inhibitory effect of anthocyanin-rich bilberry extract on  $\alpha$ -glucosidase and  $\alpha$ -amylase. *Lwt*. **145**.
- Joseph, S.V., Edirisinghe, I. and Burton-Freeman, B.M. 2016. Fruit Polyphenols: A Review of Anti-inflammatory Effects in Humans. *Critical Reviews in Food Science and Nutrition*. **56**(3), pp.419-444.
- Kafeshani, M., Entezari, M.H., Karimian, J., Pourmasoumi, M., Maracy, M.R., Amini, M.R. and Hadi, A. 2017. A comparative study of the effect of green tea and sour tea on blood pressure and lipid profile in healthy adult men. *ARYA atherosclerosis*. **13**(3), p109.

- Kahn, S.E., Hull, R.L. and Utzschneider, K.M. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. **444**(7121), pp.840-846.
- Kalita, D., Holm, D.G., LaBarbera, D.V., Petrash, J.M. and Jayanty, S.S. 2018. Inhibition of alpha-glucosidase, alpha-amylase, and aldose reductase by potato polyphenolic compounds. *PLoS One*. **13**(1), pe0191025.
- Kalt, W., Cassidy, A., Howard, L.R., Krikorian, R., Stull, A.J., Tremblay, F. and Zamora-Ros, R. 2020. Recent Research on the Health Benefits of Blueberries and Their Anthocyanins. *Advances in Nutrition*. **11**(2), pp.224-236.
- Kao, E.-S., Tseng, T.-H., Lee, H.-J., Chan, K.-C. and Wang, C.-J. 2009. Anthocyanin extracted from Hibiscus attenuate oxidized LDL-mediated foam cell formation involving regulation of CD36 gene. *Chemico-Biological Interactions*. **179**(2), pp.212-218.
- Kao, E.S., Yang, M.Y., Hung, C.H., Huang, C.N. and Wang, C.J. 2016. Polyphenolic extract from Hibiscus sabdariffa reduces body fat by inhibiting hepatic lipogenesis and preadipocyte adipogenesis. *Food & Function*. **7**(1), pp.171-182.
- Karkute, G.S., Koley, K.T., Yengkhom, K.B., Tripathi, A., Srivastava, S., Maurya, A. and Singh, B. 2018. Anti-diabetic Phenolic Compounds of Black Carrot (*Daucus carota* Subspecies *sativus* var. *atrorubens* Alef.) Inhibit Enzymes of Glucose Metabolism: An in silico and in vitro Validation. *Medicinal Chemistry*. **14**(6), pp.641-649.
- Kerimi, A., Nyambe-Silavwe, H., Gauer, J.S., Tomás-Barberán, F.A. and Williamson, G. 2017. Pomegranate juice, but not an extract, confers a lower glycemic response on a high-glycemic index food: randomized, crossover, controlled trials in healthy subjects. *American Journal of Clinical Nutrition*. **106**(6), pp.1384-1393.
- Khadayat, K., Marasini, B.P., Gautam, H., Ghaju, S. and Parajuli, N. 2020. Evaluation of the alpha-amylase inhibitory activity of Nepalese medicinal plants used in the treatment of diabetes mellitus. *Clinical Phytoscience*. **6**(1).
- Khoo, H.E., Azlan, A., Tang, S.T. and Lim, S.M. 2017. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & Nutrition Research*. **61**(1), pp.1361779-1361779.
- Kim, J.K., So, H., Youn, M.J., Kim, H.J., Kim, Y., Park, C., Kim, S.J., Ha, Y.A., Chai, K.Y., Kim, S.M., Kim, K.Y. and Park, R. 2007. Hibiscus sabdariffa L. water extract inhibits the adipocyte differentiation through the PI3-K and MAPK pathway. *Journal of Ethnopharmacology*. **114**(2), pp.260-267.
- Kim, J.S., Kwon, C.S. and Son, K.H. 2000. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology, and Biochemistry*. **64**(11), pp.2458-2461.
- Kim, Y., Keogh, J.B. and Clifton, P.M. 2016. Polyphenols and Glycemic Control. *Nutrients*. **8**(1).
- Konczak, I. and Zhang, W. 2004. Anthocyanins—more than nature's colours. *Journal of Biomedicine and Biotechnology*. **2004**(5), p239.
- Krga, I. and Milenkovic, D. 2019. Anthocyanins: From Sources and Bioavailability to Cardiovascular-Health Benefits and Molecular Mechanisms of Action. *Journal of Agricultural and Food Chemistry*. **67**(7), pp.1771-1783.
- Le Floch, J.P., Escuyer, P., Baudin, E., Baudon, D. and Perlemuter, L. 1990. Blood glucose area under the curve. Methodological aspects. *Diabetes Care*. **13**(2), pp.172-175.
- Leahy, J.L., Aleppo, G., Fonseca, V.A., Garg, S.K., Hirsch, I.B., McCall, A.L., McGill, J.B. and Polonsky, W.H. 2019. Optimizing Postprandial Glucose Management in Adults With Insulin-Requiring Diabetes: Report and Recommendations. *Journal of the Endocrine Society*. **3**(10), pp.1942-1957.

- Lee, J., Durst, R.W., Wrolstad, R.E. and Collaborators:. 2019. Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study. *Journal of AOAC International*. **88**(5), pp.1269-1278.
- Lee, W.-C., Wang, C.-J., Chen, Y.-H., Hsu, J.-D., Cheng, S.-Y., Chen, H.-C. and Lee, H.-J. 2009. Polyphenol Extracts from Hibiscus sabdariffa Linnaeus Attenuate Nephropathy in Experimental Type 1 Diabetes. *Journal of Agricultural and Food Chemistry*. **57**(6), pp.2206-2210.
- Lenzen, S. 2008. Oxidative stress: the vulnerable beta-cell. *Biochemical Society Transactions*. **36**(Pt 3), pp.343-347.
- Les, F., Casedas, G., Gomez, C., Moliner, C., Valero, M.S. and Lopez, V. 2021. The role of anthocyanins as antidiabetic agents: from molecular mechanisms to in vivo and human studies. *Journal of Physiology and Biochemistry* **77**(1), pp.109-131.
- Les, F., Cásedas, G., Gómez, C., Moliner, C., Valero, M.S. and López, V. 2021. The role of anthocyanins as antidiabetic agents: from molecular mechanisms to in vivo and human studies. *Journal of Physiology and Biochemistry*. **77**(1), pp.109-131.
- Li, D., Wang, P., Luo, Y., Zhao, M. and Chen, F. 2017. Health benefits of anthocyanins and molecular mechanisms: Update from recent decade. *Critical Reviews in Food Science and Nutrition*. **57**(8), pp.1729-1741.
- Lin, T.-L., Lin, H.-H., Chen, C.-C., Lin, M.-C., Chou, M.-C. and Wang, C.-J. 2007. Hibiscus sabdariffa extract reduces serum cholesterol in men and women. *Nutrition Research*. **27**(3), pp.140-145.
- Liu, D., Moberg, E., Kollind, M., Lins, P.E., Adamson, U. and Macdonald, I.A. 1992. Arterial, arterialized venous, venous and capillary blood glucose measurements in normal man during hyperinsulinaemic euglycaemia and hypoglycaemia. *Diabetologia*. **35**(3), pp.287-290.
- Ma, L., Sun, Z., Zeng, Y., Luo, M. and Yang, J. 2018. Molecular Mechanism and Health Role of Functional Ingredients in Blueberry for Chronic Disease in Human Beings. *International Journal of Molecular Sciences*. **19**(9).
- Mane, C., Loonis, M., Juhel, C., Dufour, C. and Malien-Aubert, C. 2011. Food Grade Lingonberry Extract: Polyphenolic Composition and In Vivo Protective Effect against Oxidative Stress. *Journal of Agricultural and Food Chemistry*. **59**(7), pp.3330-3339.
- Mardiah, Z.F., Prangdimurti, E. and Damanik, R. 2014. The effect of roselle extract (Hibiscus sabdariffa Linn.) on blood glucose level and total antioxidant level on diabetic rat induced by streptozotocin. *IOSR Journal of Pharmacy*. **4**(10), pp.8-16.
- Martinez-Gonzalez, A.I., Diaz-Sanchez, A.G., de la Rosa, L.A., Bustos-Jaimes, I. and Alvarez-Parrilla, E. 2019. Inhibition of alpha-amylase by flavonoids: Structure activity relationship (SAR). *Spectrochimica Acta. Part A: Molecular and Biomolecular Spectroscopy*. **206**, pp.437-447.
- Mayasari, N.R., Susetyowati, Wahyuningsih, M.S.H. and Probosuseno. 2018. Antidiabetic Effect of Rosella-Stevia Tea on Prediabetic Women in Yogyakarta, Indonesia. *Journal of the American College of Nutrition*. **37**(5), pp.373-379.
- McDougall, G.J., Martinussen, I. and Stewart, D. 2008. Towards fruitful metabolomics: High throughput analyses of polyphenol composition in berries using direct infusion mass spectrometry. *Journal of Chromatography B*. **871**(2), pp.362-369.
- McDougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A. and Stewart, D. 2005. Different polyphenolic components of soft fruits inhibit alpha-amylase and alpha-glucosidase. *Journal of Agricultural and Food Chemistry*. **53**(7), pp.2760-2766.
- McDougall, G.J. and Stewart, D. 2005. The inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors*. **23**(4), pp.189-195.

- McKay, D.L., Chen, C.Y., Saltzman, E. and Blumberg, J.B. 2010. Hibiscus sabdariffa L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults. *Journal of Nutrition*. **140**(2), pp.298-303.
- Michalska, A. and Lysiak, G. 2015. Bioactive Compounds of Blueberries: Post-Harvest Factors Influencing the Nutritional Value of Products. *International Journal of Molecular Sciences*. **16**(8), pp.18642-18663.
- Miller, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*. **31**(3), pp.426-428.
- Mojiminiyi, F., Dikko, M., Muhammad, B., Ojobor, P., Ajagbonna, O., Okolo, R., Igbokwe, U., Mojiminiyi, U., Fagbemi, M. and Bello, S. 2007. Antihypertensive effect of an aqueous extract of the calyx of Hibiscus sabdariffa. *Fitoterapia*. **78**(4), pp.292-297.
- Mousavinejad, G., Emam-Djomeh, Z., Rezaei, K. and Khodaparast, M.H.H. 2009. Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars. *Food Chemistry*. **115**(4), pp.1274-1278.
- Mozaffari-Khosravi, H., Jalali-Khanabadi, B.A., Afkhami-Ardekani, M., Fatehi, F. and Noori-Shadkam, M. 2009. The effects of sour tea (Hibiscus sabdariffa) on hypertension in patients with type II diabetes. *Journal of Human Hypertension*. **23**(1), pp.48-54.
- Mursu, J., Virtanen, J.K., Tuomainen, T.P., Nurmi, T. and Voutilainen, S. 2014. Intake of fruit, berries, and vegetables and risk of type 2 diabetes in Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. *American Journal of Clinical Nutrition*. **99**(2), pp.328-333.
- Murthy, P.S., Manjunatha, M., Sulochannama, G. and Naidu, M.M. 2012. Extraction, characterization and bioactivity of coffee anthocyanins. *European Journal of Biology*. **4**(1), pp.13-19.
- Nielsen, I.L.F., Haren, G.R., Magnussen, E.L., Dragsted, L.O. and Rasmussen, S.E. 2003. Quantification of Anthocyanins in Commercial Black Currant Juices by Simple High-Performance Liquid Chromatography. Investigation of Their pH Stability and Antioxidative Potency. *Journal of Agricultural and Food Chemistry*. **51**(20), pp.5861-5866.
- Nijpels, G., Boorsma, W., Dekker, J.M., Kostense, P.J., Bouter, L.M. and Heine, R.J. 2008. A study of the effects of acarbose on glucose metabolism in patients predisposed to developing diabetes: the Dutch acarbose intervention study in persons with impaired glucose tolerance (DAISI). *Diabetes/Metabolism Research and Reviews*. **24**(8), pp.611-616.
- Nizamutdinova, I.T., Jin, Y.C., Chung, J.I., Shin, S.C., Lee, S.J., Seo, H.G., Lee, J.H., Chang, K.C. and Kim, H.J. 2009. The anti-diabetic effect of anthocyanins in streptozotocin-induced diabetic rats through glucose transporter 4 regulation and prevention of insulin resistance and pancreatic apoptosis. *Molecular Nutrition & Food Research*. **53**(11), pp.1419-1429.
- Nwachukwu, D., Aneke, E., Obika, L. and Nwachukwu, N. 2015. Investigation of antihypertensive effectiveness and tolerability of Hibiscus sabdariffa in mild to moderate hypertensive subjects in Enugu, South-east, Nigeria. *American Journal of Phytomedicine and Clinical Therapeutics*. **19**(2), pp.148-152.
- Nyambe-Silavwe, H., Villa-Rodriguez, J.A., Ifie, I., Holmes, M., Aydin, E., Jensen, J.M. and Williamson, G. 2015. Inhibition of human  $\alpha$ -amylase by dietary polyphenols. *Journal of Functional Foods*. **19**, pp.723-732.
- Nyambe-Silavwe, H. and Williamson, G. 2016. Polyphenol- and fibre-rich dried fruits with green tea attenuate starch-derived postprandial blood glucose and insulin: a randomised, controlled, single-blind, cross-over intervention. *British Journal of Nutrition*. **116**(3), pp.443-450.

- Ojeda, D., Jiménez-Ferrer, E., Zamilpa, A., Herrera-Arellano, A., Tortoriello, J. and Alvarez, L. 2010. Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-O-sambubiosides from *Hibiscus sabdariffa*. *Journal of Ethnopharmacology*. **127**(1), pp.7-10.
- Ostberg-Potthoff, J.J., Berger, K., Richling, E. and Winterhalter, P. 2019. Activity-Guided Fractionation of Red Fruit Extracts for the Identification of Compounds Influencing Glucose Metabolism. *Nutrients*. **11**(5), p1166.
- Patterson, C.C., Harjutsalo, V., Rosenbauer, J., Neu, A., Cinek, O., Skrivarhaug, T., Rami-Merhar, B., Soltész, G., Svensson, J., Parslow, R.C., Castell, C., Schoenle, E.J., Bingley, P.J., Dahlquist, G., Jarosz-Chobot, P.K., Marčiulionytė, D., Roche, E.F., Rothe, U., Bratina, N., Ionescu-Tirgoviste, C., Weets, I., Kocova, M., Cherubini, V., Rojnic Putarek, N., deBeaufort, C.E., Samardzic, M. and Green, A. 2019. Trends and cyclical variation in the incidence of childhood type 1 diabetes in 26 European centres in the 25 year period 1989–2013: a multicentre prospective registration study. *Diabetologia*. **62**(3), pp.408-417.
- Peng, C.H., Chyau, C.C., Chan, K.C., Chan, T.H., Wang, C.J. and Huang, C.N. 2011. *Hibiscus sabdariffa* polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. *Journal of Agricultural and Food Chemistry*. **59**(18), pp.9901-9909.
- Podsdek, A., Majewska, I., Redzyna, M., Sosnowska, D. and Koziolkiewicz, M. 2014. In vitro inhibitory effect on digestive enzymes and antioxidant potential of commonly consumed fruits. *Journal of Agricultural and Food Chemistry*. **62**(20), pp.4610-4617.
- Proença, C., Freitas, M., Ribeiro, D., Oliveira, E.F.T., Sousa, J.L.C., Tomé, S.M., Ramos, M.J., Silva, A.M.S., Fernandes, P.A. and Fernandes, E. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico structure-activity relationship study. *Journal of Enzyme Inhibition and Medicinal Chemistry*. **32**(1), pp.1216-1228.
- Promyos, N., Temviriyankul, P. and Suttisansanee, U. 2020. Investigation of Anthocyanidins and Anthocyanins for Targeting alpha-Glucosidase in Diabetes Mellitus. *Preventive Nutrition and Food Science*. **25**(3), pp.263-271.
- Prpa, E.J., Bajka, B.H., Ellis, P.R., Butterworth, P.J., Corpe, C.P. and Hall, W.L. 2021. A systematic review of in vitro studies evaluating the inhibitory effects of polyphenol-rich fruit extracts on carbohydrate digestive enzymes activity: a focus on culinary fruits consumed in Europe. *Critical Reviews in Food Science and Nutrition*. **61**(22), pp.3783-3803.
- Putta, S., Yarla, N.S., Kumar, K.E., Lakkappa, D.B., Kamal, M.A., Scotti, L., Scotti, M.T., Ashraf, G.M., Rao, B.S.B., D, S.K., Reddy, G.V., Tarasov, V.V., Imandi, S.B. and Aliev, G. 2018. Preventive and Therapeutic Potentials of Anthocyanins in Diabetes and Associated Complications. *Current Medicinal Chemistry*. **25**(39), pp.5347-5371.
- Raptis, S.A. and Dimitriadis, G.D. 2001. Oral hypoglycemic agents: insulin secretagogues, alpha-glucosidase inhibitors and insulin sensitizers. *Experimental and Clinical Endocrinology & Diabetes*. **109 Suppl 2**, pp.S265-287.
- Rasheed, D.M., Porzel, A., Frolov, A., El Seedi, H.R., Wessjohann, L.A. and Farag, M.A. 2018. Comparative analysis of *Hibiscus sabdariffa* (roselle) hot and cold extracts in respect to their potential for alpha-glucosidase inhibition. *Food Chemistry*. **250**, pp.236-244.
- Rehani, P.R., Iftikhar, H., Nakajima, M., Tanaka, T., Jabbar, Z. and Rehani, R.N. 2019. Safety and Mode of Action of Diabetes Medications in comparison with 5-Aminolevulinic Acid (5-ALA). *Journal of diabetes research*. **2019**, pp.4267357-4267357.
- Röder, P.V., Wu, B., Liu, Y. and Han, W. 2016. Pancreatic regulation of glucose homeostasis. *Experimental and Molecular Medicine*. **48**(3), pp.e219-e219.

- Roy, M., Sen, S. and Chakraborti, A.S. 2008. Action of pelargonidin on hyperglycemia and oxidative damage in diabetic rats: implication for glycation-induced hemoglobin modification. *Life Sciences*. **82**(21-22), pp.1102-1110.
- Sachdewa, A. and Khemani, L.D. 2003. Effect of Hibiscus rosa sinensis Linn. ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. *Journal of Ethnopharmacology*. **89**(1), pp.61-66.
- Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., Colagiuri, S., Guariguata, L., Motala, A.A., Ogurtsova, K., Shaw, J.E., Bright, D., Williams, R. and Committee, I.D.F.D.A. 2019. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. *Diabetes Research and Clinical Practice*. **157**, p107843.
- Sagandira, C.R., Khasipo, A.Z., Sagandira, M.B. and Watts, P. 2021. An overview of the synthetic routes to essential oral anti-diabetes drugs. *Tetrahedron*. **96**, p132378.
- Sancho, R.A.S. and Pastore, G.M. 2012. Evaluation of the effects of anthocyanins in type 2 diabetes. *Food Research International*. **46**(1), pp.378-386.
- Sartipy, P. and Loskutoff, D.J. 2003. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*. **100**(12), pp.7265-7270.
- Sasaki, R., Nishimura, N., Hoshino, H., Isa, Y., Kadowaki, M., Ichi, T., Tanaka, A., Nishiumi, S., Fukuda, I., Ashida, H., Horio, F. and Tsuda, T. 2007. Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to downregulation of retinol binding protein 4 expression in diabetic mice. *Biochemical Pharmacology*. **74**(11), pp.1619-1627.
- Scalbert, A., Manach, C., Morand, C., Rémésy, C. and Jiménez, L. 2005. Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition*. **45**(4), pp.287-306.
- Scazzocchio, B., Vari, R., Filesi, C., D'Archivio, M., Santangelo, C., Giovannini, C., Iacovelli, A., Silecchia, G., Li Volti, G., Galvano, F. and Masella, R. 2011. Cyanidin-3-O- $\beta$ -glucoside and protocatechuic acid exert insulin-like effects by upregulating PPAR $\gamma$  activity in human omental adipocytes. *Diabetes*. **60**(9), pp.2234-2244.
- Schulze, C., Bangert, A., Kottra, G., Geillinger, K.E., Schwanck, B., Vollert, H., Blaschek, W. and Daniel, H. 2014. Inhibition of the intestinal sodium-coupled glucose transporter 1 (SGLT1) by extracts and polyphenols from apple reduces postprandial blood glucose levels in mice and humans. *Molecular Nutrition & Food Research*. **58**(9), pp.1795-1808.
- Seeram, N.P. 2012. Emerging research supporting the positive effects of berries on human health and disease prevention. *Journal of Agricultural and Food Chemistry*. **60**(23), pp.5685-5686.
- Serrano, M., Guillén, F., Martínez-Romero, D., Castillo, S. and Valero, D. 2005. Chemical Constituents and Antioxidant Activity of Sweet Cherry at Different Ripening Stages. *Journal of Agricultural and Food Chemistry*. **53**(7), pp.2741-2745.
- Seymour, E.M., Singer, A.A., Kirakosyan, A., Urcuyo-Llanes, D.E., Kaufman, P.B. and Bolling, S.F. 2008. Altered hyperlipidemia, hepatic steatosis, and hepatic peroxisome proliferator-activated receptors in rats with intake of tart cherry. *Journal of Medicinal Food*. **11**(2), pp.252-259.
- Sindi, H.A., Marshall, L.J. and Morgan, M.R. 2014. Comparative chemical and biochemical analysis of extracts of Hibiscus sabdariffa. *Food Chemistry*. **164**, pp.23-29.
- Singleton, V.L., Orthofer, R. and Lamuela-Raventós, R.M. 1999. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*. **299**, pp.152-178.

- Slaughter, S.L., Ellis, P.R. and Butterworth, P.J. 2001. An investigation of the action of porcine pancreatic  $\alpha$ -amylase on native and gelatinised starches. *Biochimica et Biophysica Acta (BBA) - General Subjects*. **1525**(1), pp.29-36.
- Spinardi, A., Cola, G., Gardana, C.S. and Mignani, I. 2019. Variation of Anthocyanin Content and Profile Throughout Fruit Development and Ripening of Highbush Blueberry Cultivars Grown at Two Different Altitudes. *Frontiers in Plant Science*. **10**(1045).
- Stull, A.J. 2016. Blueberries' Impact on Insulin Resistance and Glucose Intolerance. *Antioxidants (Basel)*. **5**(4).
- Stull, A.J., Cash, K.C., Johnson, W.D., Champagne, C.M. and Cefalu, W.T. 2010. Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. *Journal of Nutrition*. **140**(10), pp.1764-1768.
- Sudhir, R. and Mohan, V. 2002. Postprandial hyperglycemia in patients with type 2 diabetes mellitus. *Treatments in Endocrinology*. **1**(2), pp.105-116.
- Sui, X., Zhang, Y. and Zhou, W. 2016. In vitro and in silico studies of the inhibition activity of anthocyanins against porcine pancreatic  $\alpha$ -amylase. *Journal of Functional Foods*. **21**, pp.50-57.
- Tadera, K., Minami, Y., Takamatsu, K. and Matsuoka, T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *Journal of Nutritional Science*. **52**(2), pp.149-153.
- Takikawa, M., Inoue, S., Horio, F. and Tsuda, T. 2010. Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. *Journal of Nutrition*. **140**(3), pp.527-533.
- Tan, Y. and Chang, S.K. 2017. Digestive enzyme inhibition activity of the phenolic substances in selected fruits, vegetables and tea as compared to black legumes. *Journal of Functional Foods*. **38**, pp.644-655.
- Tonutare, T., Moor, U. and Szajdak, L. 2014. Strawberry anthocyanin determination by pH differential spectroscopic method-how to get true results. *Acta Scientiarum Polonorum Hortorum Cultus*. **13**, pp.35-47.
- Torronen, R., Kolehmainen, M., Sarkkinen, E., Mykkanen, H. and Niskanen, L. 2012. Postprandial glucose, insulin, and free fatty acid responses to sucrose consumed with blackcurrants and lingonberries in healthy women. *American Journal of Clinical Nutrition*. **96**(3), pp.527-533.
- Torronen, R., Kolehmainen, M., Sarkkinen, E., Poutanen, K., Mykkanen, H. and Niskanen, L. 2013. Berries reduce postprandial insulin responses to wheat and rye breads in healthy women. *Journal of Nutrition*. **143**(4), pp.430-436.
- Torronen, R., Sarkkinen, E., Tapola, N., Hautaniemi, E., Kilpi, K. and Niskanen, L. 2010. Berries modify the postprandial plasma glucose response to sucrose in healthy subjects. *British Journal of Nutrition*. **103**(8), pp.1094-1097.
- Tripathi, B.K. and Srivastava, A.K. 2006. Diabetes mellitus: complications and therapeutics. *Medical Science Monitor*. **12**(7), pp.130-147.
- Tseng, T.-H., Kao, T.-W., Chu, C.-Y., Chou, F.-P., Lin, W.-L. and Wang, C.-J. 2000. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. *Biochemical Pharmacology*. **60**(3), pp.307-315.
- Tseng, T.-H., Wang, C.-J. and Kao, E.-S. 1996. Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoxide in rat primary hepatocytes. *Chemico-Biological Interactions*. **101**(2), pp.137-148.

- Tseng, T.H., Kao, E.S., Chu, C.Y., Chou, F.P., Lin Wu, H.W. and Wang, C.J. 1997. Protective effects of dried flower extracts of *Hibiscus sabdariffa* L. against oxidative stress in rat primary hepatocytes. *Food and Chemical Toxicology*. **35**(12), pp.1159-1164.
- Tsuda, T., Horio, F., Uchida, K., Aoki, H. and Osawa, T. 2003. Dietary Cyanidin 3-O- $\beta$ -D-Glucoside-Rich Purple Corn Color Prevents Obesity and Ameliorates Hyperglycemia in Mice. *The Journal of Nutrition*. **133**(7), pp.2125-2130.
- Turina, M., Christ-Crain, M. and Polk, H.C.J. 2006. Diabetes and hyperglycemia: Strict glycemic control. *Critical Care Medicine*. **34**(9), pp.S291-S300.
- Turrini, E., Ferruzzi, L. and Fimognari, C. 2017. Possible Effects of Dietary Anthocyanins on Diabetes and Insulin Resistance. *Current Drug Targets*. **18**(6), pp.629-640.
- Ueno, H., Tsuchimochi, W., Wang, H.W., Yamashita, E., Tsubouchi, C., Nagamine, K., Sakoda, H. and Nakazato, M. 2015. Effects of Miglitol, Acarbose, and Sitagliptin on Plasma Insulin and Gut Peptides in Type 2 Diabetes Mellitus: A Crossover Study. *Diabetes Therapy*. **6**(2), pp.187-196.
- UKPDS. 1998. Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 39. UK Prospective Diabetes Study Group. *British Medical Journal*. **317**(7160), pp.713-720.
- Vendrame, S., Del Bo, C., Ciappellano, S., Riso, P. and Klimis-Zacas, D. 2016. Berry Fruit Consumption and Metabolic Syndrome. *Antioxidants (Basel)*. **5**(4).
- Venn, B.J., Kataoka, M. and Mann, J. 2014. The use of different reference foods in determining the glycemic index of starchy and non-starchy test foods. *Nutrition Journal*. **13**(1), p50.
- Vinson, J.A., Bose, P., Proch, J., Al Kharrat, H. and Samman, N. 2008. Cranberries and cranberry products: powerful in vitro, ex vivo, and in vivo sources of antioxidants. *Journal of Agricultural and Food Chemistry*. **56**(14), pp.5884-5891.
- Viollet, B., Lantier, L., Devin-Leclerc, J., Hebrard, S., Amouyal, C., Mounier, R., Foretz, M. and Andreelli, F. 2009. Targeting the AMPK pathway for the treatment of Type 2 diabetes. *Frontiers in bioscience (Landmark edition)*. **14**, pp.3380-3400.
- Visvanathan, R., Houghton, M.J. and Williamson, G. 2021. Maltoheptaoside hydrolysis with chromatographic detection and starch hydrolysis with reducing sugar analysis: Comparison of assays allows assessment of the roles of direct alpha-amylase inhibition and starch complexation. *Food Chemistry*. **343**, p128423.
- Visvanathan, R., Qader, M., Jayathilake, C., Jayawardana, B.C., Liyanage, R. and Sivakanesan, R. 2020. Critical review on conventional spectroscopic alpha-amylase activity detection methods: merits, demerits, and future prospects. *Journal of the Science of Food and Agriculture*. **100**(7), pp.2836-2847.
- Vita, J.A. 2005. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. *The American journal of clinical nutrition*. **81**(1), pp.292S-297S.
- Wahabi, H.A., Alansary, L.A., Al-Sabban, A.H. and Glasziou, P. 2010. The effectiveness of *Hibiscus sabdariffa* in the treatment of hypertension: a systematic review. *Phytomedicine*. **17**(2), pp.83-86.
- Wallace, T.C. 2011. Anthocyanins in cardiovascular disease. *Advances in Nutrition*. **2**(1), pp.1-7.
- Wallace, T.C. and Giusti, M.M. 2015. Anthocyanins. *Advances in Nutrition*. **6**(5), pp.620-622.
- Wang, P.-Y., Fang, J.-C., Gao, Z.-H., Zhang, C. and Xie, S.-Y. 2016. Higher intake of fruits, vegetables or their fiber reduces the risk of type 2 diabetes: A meta-analysis. *Journal of diabetes investigation*. **7**(1), pp.56-69.

- Wedick, N.M., Pan, A., Cassidy, A., Rimm, E.B., Sampson, L., Rosner, B., Willett, W., Hu, F.B., Sun, Q. and van Dam, R.M. 2012. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. *American Journal of Clinical Nutrition*. **95**(4), pp.925-933.
- Williamson, G. 2013. Possible effects of dietary polyphenols on sugar absorption and digestion. *Molecular Nutrition & Food Research*. **57**(1), pp.48-57.
- Wilson, T., Singh, A.P., Vorsa, N., Goettl, C.D., Kittleson, K.M., Roe, C.M., Kastello, G.M. and Ragsdale, F.R. 2008. Human glycemic response and phenolic content of unsweetened cranberry juice. *Journal of Medicinal Food*. **11**(1), pp.46-54.
- Wolever, T.M.S., Vorster, H.H., Björck, I., Brand-Miller, J., Brighenti, F., Mann, J.I., Ramdath, D.D., Granfeldt, Y., Holt, S., Perry, T.L., Venter, C. and Xiaomei, W. 2003. Determination of the glycaemic index of foods: interlaboratory study. *European Journal of Clinical Nutrition*. **57**(3), pp.475-482.
- Wong, P.K., Yusof, S., Ghazali, H.M. and Che Man, Y.B. 2002. Physico-chemical characteristics of roselle (*Hibiscus sabdariffa*L.). *Nutrition & Food Science*. **32**(2), pp.68-73.
- Xie, L., Mo, J., Ni, J., Xu, Y., Su, H., Xie, J. and Chen, W. 2020. Structure-based design of human pancreatic amylase inhibitors from the natural anthocyanin database for type 2 diabetes. *Food and Function*. **11**(4), pp.2910-2923.
- Xu, Y., Xie, L., Xie, J., Liu, Y. and Chen, W. 2019. Pelargonidin-3-O-rutinoside as a novel  $\alpha$ -glucosidase inhibitor for improving postprandial hyperglycemia. *Chemical Communications*. **55**(1), pp.39-42.
- Yang, C., Chang, C. and Lin, J. 2012. A comparison between venous and finger-prick blood sampling on values of blood glucose. *International Proceedings of Chemical, Biological and Environmental Engineering*. **39**, pp.206-210.
- Yang, M.Y., Peng, C.H., Chan, K.C., Yang, Y.S., Huang, C.N. and Wang, C.J. 2010. The hypolipidemic effect of *Hibiscus sabdariffa* polyphenols via inhibiting lipogenesis and promoting hepatic lipid clearance. *Journal of Agricultural and Food Chemistry*. **58**(2), pp.850-859.
- You, Q., Chen, F., Wang, X., Luo, P.G. and Jiang, Y. 2011. Inhibitory effects of muscadine anthocyanins on alpha-glucosidase and pancreatic lipase activities. *Journal of Agricultural and Food Chemistry*. **59**(17), pp.9506-9511.
- Yousuf, B., Gul, K., Wani, A.A. and Singh, P. 2016. Health Benefits of Anthocyanins and Their Encapsulation for Potential Use in Food Systems: A Review. *Crit Rev Food Sci Nutr*. **56**(13), pp.2223-2230.
- Zhang, A.J., Rimando, A.M., Mizuno, C.S. and Mathews, S.T. 2017. alpha-Glucosidase inhibitory effect of resveratrol and piceatannol. *Journal of Nutritional Biochemistry*. **47**, pp.86-93.
- Zhang, B., Kang, M., Xie, Q., Xu, B., Sun, C., Chen, K. and Wu, Y. 2011. Anthocyanins from Chinese bayberry extract protect  $\beta$  cells from oxidative stress-mediated injury via HO-1 upregulation. *Journal of agricultural and food chemistry*. **59**(2), pp.537-545.
- Zhang, B.W., Li, X., Sun, W.L., Xing, Y., Xiu, Z.L., Zhuang, C.L. and Dong, Y.S. 2017. Dietary Flavonoids and Acarbose Synergistically Inhibit alpha-Glucosidase and Lower Postprandial Blood Glucose. *Journal of Agricultural and Food Chemistry*. **65**(38), pp.8319-8330.
- Zhang, L., Li, J., Hogan, S., Chung, H., Welbaum, G.E. and Zhou, K. 2010. Inhibitory effect of raspberries on starch digestive enzyme and their antioxidant properties and phenolic composition. *Food Chemistry*. **119**(2), pp.592-599.
- Zulfiqar, S., Benton, K., Hassan, T., Marshall, L. and Boesch, C. 2019. In vitro and in vivo anti-diabetic properties of *Hibiscus sabdariffa*. *Proceedings of the Nutrition Society*. **78**(OCE2), pE59.



## List of appendixes

### Appendix A

#### Study: Effect of Hibiscus drink on glycaemic response in healthy volunteers

Date: / / participant code.....

Please provide brief information about yourself by ticking the appropriate answer (s) or provide additional information where necessary.

1. Would you consider yourself to be in good health?

Yes       No

If the answer is 'no', please explain the reasons for your choice:

.....

2. Have you ever been told by a doctor or other health professional that you have any of the following?

<input type="checkbox"/> Type I diabetes	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know
<input type="checkbox"/> Type II diabetes	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know
<input type="checkbox"/> Gestational diabetes (Diabetes during pregnancy)	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know
<input type="checkbox"/> Prediabetes	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know
<input type="checkbox"/> Impaired fasting glucose	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know
<input type="checkbox"/> Impaired glucose tolerance	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know
<input type="checkbox"/> Borderline diabetes	<input type="checkbox"/> Yes	<input type="checkbox"/> No	<input type="checkbox"/> don't know

3. Do you suffer from any health problems associated with your digestive system (stomach, intestine), your pancreas, your liver or your kidneys?

Yes       No       don't know

If the answer is 'yes', then please provide details of your condition.

.....

4. Are you taking any type of medication?

Yes       No

If the answer is 'yes', please list below the name of all the medications that you are currently taking:

.....

5. Do you have any history of allergy (food or non-food related)?

Yes No

If yes, which kind of allergy do you have?

.....

6. Are you gluten intolerant?

Yes No  don't know

7. Are you pregnant? Yes No  don't know

8. Are you breastfeeding? Yes No

*Thank you for your time, the answers will allow us to determine whether you are eligible for the trial. A researcher will contact you soon to explain what the next steps are.*

## Appendix B

### Pre-study questionnaire

#### Study: Effect of Hibiscus drink on glycaemic response in healthy volunteers

Date: / / participant code.....

Please provide brief information about yourself by tick at the appropriate answer (s) or provide additional information where necessary. These questions are designed to gather information about demographic, diet and lifestyle factors that may affect how you respond to food.

Nationality: <input type="checkbox"/> United kingdom <input type="checkbox"/> EU <input type="checkbox"/> Other.....	Weight:
Age (years):	Height:
Gender: Male <input type="checkbox"/> Female <input type="checkbox"/>	Waist circumference:

1. Ethnic background :

- White - British
- White - Irish
- White – Scottish
- Irish Traveller
- Other White Background
- Black or Black British– Caribbean
- Black or Black British – African
- Other Black Background
- Asian or Asian British - Indian
- Asian or Asian British - Pakistani
- Asian or Asian British – Bangladeshi
- Chinese
- Asian Other
- Mixed – White and Black Caribbean
- Mixed – White and Black African
- Mixed – White and Asian
- Other Mixed Background
- Other Ethnic Background
- Information Refused

2. Do you consider yourself to be in good health today?

- Yes No

If the answer is 'no', please explain the reasons for your choice:

.....

3. Do you drink alcohol?

Yes No

If the answer is 'yes', how many units do you consume every week?  
(1 unit is ½ glass of wine, ½ pint of beer, 1 measure of spirits)

.....

4. Are you currently on a special of diet?

Yes No

If the answer is 'yes', please specify what kind of diet you are on:

.....

5. Are you vegetarian or vegan?

Yes No

6. Do you smoke cigarettes/cigars/pipe regularly?

Yes No

If the answer is 'yes', how many do you smoke in our day? .....

7. How did you come to the university today?

by foot  by bicycle

by any other transportation (please specify.....)

8. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?

\_\_\_\_\_ days per week

No vigorous physical activities  Skip to question 3

9. How much time did you usually spend doing vigorous physical activities on one of those days?

\_\_\_\_\_ hours per day \_\_\_\_\_ minutes per day

Don't know/Not sure

10. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

\_\_\_\_\_ days per week

No moderate physical activities -  Skip to question 5

11. How much time did you usually spend doing moderate physical activities on one of those days?

\_\_\_\_\_ hours per day \_\_\_\_\_ minutes per day

Don't know/Not sure

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

\_\_\_\_\_ days per week

No walking  Skip to question 7

13. . How much time did you usually spend walking on one of those days?

\_\_\_\_\_ hours per day \_\_\_\_\_ minutes per day

Don't know/Not sure

14. . During the last 7 days, how much time did you spend sitting on a weekday?

\_\_\_\_\_ hours per day \_\_\_\_\_ minutes per day

Don't know/Not sure

15. 24-hours food consumption recall

Food intake	Time	Description	amount
Break fast			
Snack			
Lunch			
Snack			
Dinner			
Snack			

*Thank you for your time*

## **Appendix C**

### **Volunteer Information Sheet**

#### **Study: Effect of Hibiscus drink on glycaemic response in healthy volunteers**

You are invited to take part in a research study at the University of Leeds. Before you decide to take part, it is important that you understand the purpose of the study and what the study involves. Please take time to read through the following information carefully. If you are unclear about anything or would like more information, please feel free to ask us. Take time to decide whether or not you wish to participate.

#### **What is the purpose of the study?**

The aim of this study is to investigate the effect of hibiscus on the glycaemic response profile. Glycaemic response is the change in blood glucose levels following ingestion of a carbohydrate-rich food such as white bread. In diabetic patients, a carbohydrate-rich diet can have detrimental effects on glycaemic control. Controlling postprandial hyperglycaemia is thought to be an important factor in the prevention and treatment of type-2 diabetes.

Hibiscus has been associated with health benefits such as lowering of blood pressure. However, there is lack of information regarding the anti-diabetic property of Hibiscus. This study will investigate if consuming hibiscus concentrate has an effect on blood glucose response to a carbohydrate-rich food. A role in blood glucose control has been suggested for a number of plant bioactives, such as polyphenols, which can be found in teas.

#### **Am I suitable to take part?**

We are looking for healthy individuals (female and male) aged 20- 50 years, living in the area of Leeds, who are not pregnant or lactating women, not allergic to any food, not diagnosed with chronic disease such as diabetes, cardiovascular disease, cancer and not taking any medication that may affect digestion or the response to glucose. We will ask about your health in a short questionnaire. If you are suitable based on the questionnaire, you will be invited to

take part in the study and come to the human study room in the Parkinson building at the University of Leeds.

### **What will the study involve?**

If you decide to take part, you will be invited to a briefing session where the researcher will explain the details of the study and answer any questions that you may have. You will be asked to provide your written consent. Participation in the study will encompass altogether 3 visits with at least 2-3 days in between each visit. On your first visit you will be asked to fill in a questionnaire regarding general intake of fruit and vegetables and antioxidant supplements and your physical activity. We will measure your height and weight.

On each visit, you will be asked to consume a carbohydrate portion (e.g. white bread) in combination with our test products in a randomized order. On the day before each visit, you will be asked to restrict your physical activity, refrain from drinking alcohol and consumption of a heavy meal at dinner time. You will be asked to fast from 10pm onwards (except water) until you arrive at the research centre. We will measure your blood glucose using finger prick glucose testing strip. You will then be asked to consume the test product with the carbohydrate portion and we will measure your blood glucose changes over the next 3 hours in regular intervals (15, 30, 45, 60, 120, 150 and after 180min). Besides glucose, we will collect blood drops into a separate small tube (100uL) in order to measure your insulin levels. After completion of the study, a £15 retail voucher will be offered as compensation for your time.

### **What are the benefits and risks of taking part in the study?**

*Benefits* - There are no direct benefits to participants taking part in the study but your participation in this research will help to expand our knowledge on the health benefits of herbal teas. We will let you know the results of your blood glucose test. If we find abnormal results, we will encourage you to discuss them with your GP.

*Risks* - You might experience temporarily sore fingers after finger pricks and there is a small risk of infection.

This research will help expanding our knowledge on the health benefits of herbal teas and may open dietary strategies for people who need to manage their blood glucose.

**Can I withdraw from the study at any time?**

Yes, volunteers are free to withdraw from the study at any time without reason. The consent form that you sign prior to entry on the study is in no way binding. It simply assures that you have read the information sheet, and that you are happy to proceed and willing to provide finger prick blood samples. Before you begin, or during the study please feel free to ask the investigator any questions you might have on any aspect of the investigation.

**How confidential are the results?**

All data and results are kept strictly confidential and each participant will only be referred to using a unique identifier code. Data collected as part of this study will be kept in a repository. Anonymous findings may be published in a scientific journal or presented at scientific meetings. If you are interested in receiving information about the findings of the study, please let us know and we will send you a copy of the research findings.

**To volunteer or for further information please contact:**

**Sadia Zulfiqar** ([fssz@leeds.ac.uk](mailto:fssz@leeds.ac.uk))

**Supervisor: Dr Christine Bosch** ([c.bosch@leeds.ac.uk](mailto:c.bosch@leeds.ac.uk)) 0113-34-30268

This study has been reviewed by The Faculty of Mathematics and Physical Sciences and Engineering Ethics Committee, University of Leeds. [MEEC 16-028]

## Appendix D

### Consent form

#### Effect of Hibiscus drink on glycaemic response in healthy volunteers

I CONFIRM THAT (please add your initials next to each statement if you agree):

I have read and understood the "Volunteer Information" sheet for the above study	
I have had the opportunity to ask questions, discuss the study and have received satisfactory answers to all my questions	
I understand that the information generated will be anonymous and that no individual result will be published	
I understand that I am free to withdraw from the project at any time without giving a reason for withdrawing and without affecting future support	
I understand that relevant sections of the data collected during the study, may be looked at by auditors from the University of Leeds or from regulatory authorities where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records	
I understand that the data collected from me to be kept in a repository and potentially be used in relevant future research in an anonymized form	
I understand that I will be asked to provide finger prick blood samples	
I agree to take part in the study and will inform the lead researcher should my contact details change	

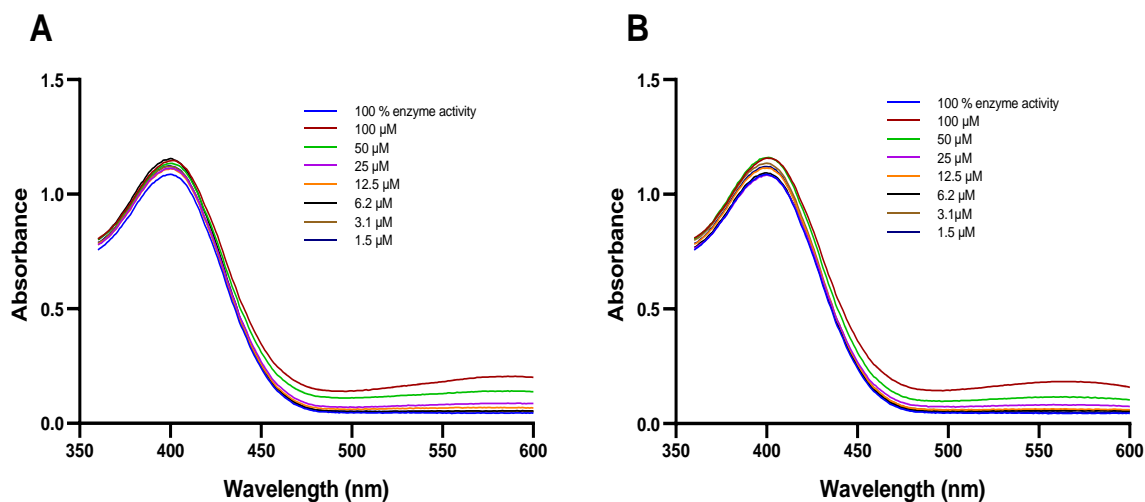
\_\_\_\_\_  
Name of Volunteer                      Date                      Signature

\_\_\_\_\_  
Name of person taking consent                      Date                      Signature\*

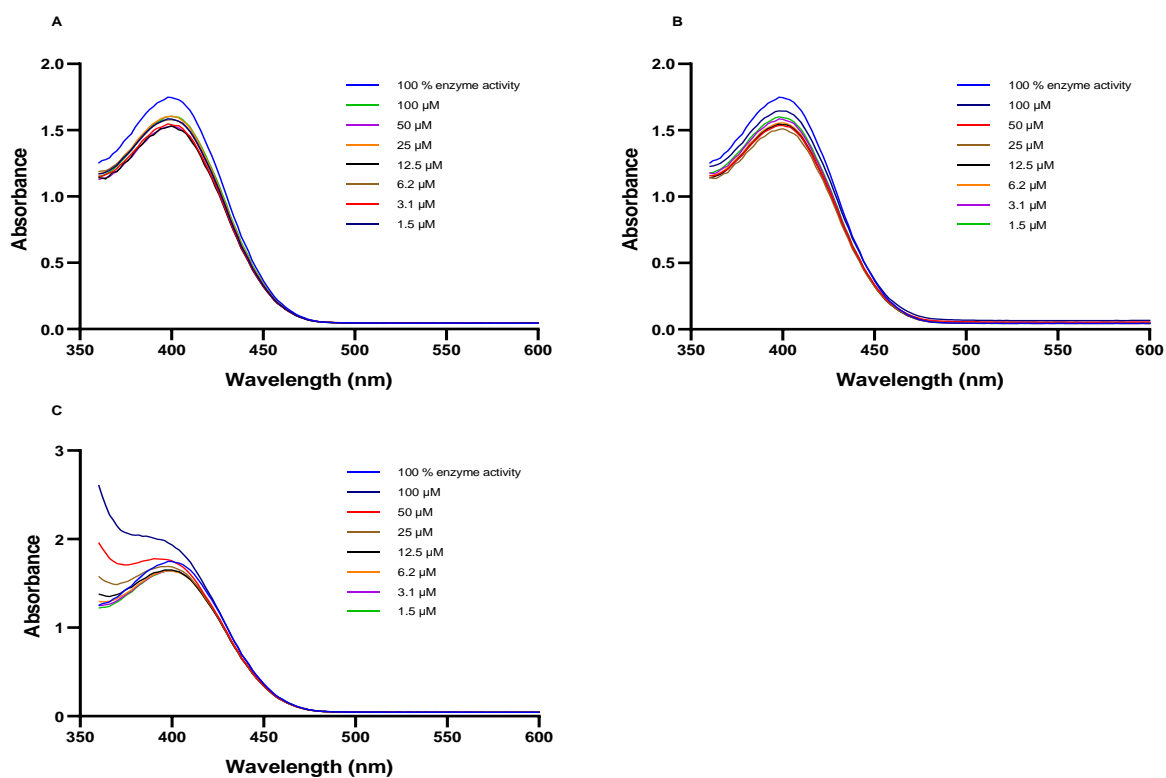
\*To be signed and dated in the presence of the participant.

Once this has been signed by all parties the participant should receive a copy of the signed and dated participant consent form, the letter/ pre-written script/ information sheet and any other written information provided to the participants. A copy of the signed and dated consent form should be kept with the project's main documents which must be kept in a secure location.

## Appendix E



**Figure 3.1 A** Wavelength scan (350-600) following  $\alpha$ -glucosidase enzyme reaction with delphinidin sambubioside (A) and cyanidin sambubioside (B) as potential inhibitors.



**Figure 3.1 B** Wavelength scan (350-600) following  $\alpha$ -glucosidase enzyme reaction with protocatechuic acid (A), gallic acid (B), and chlorogenic acid as potential inhibitors.