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Hibiscus sabdariffa inhibits α-glucosidase activity *in vitro* and lowers postprandial blood glucose response in humans

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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 α-amylase and α-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 α-glucosidase (IC_{50} 120.9 μg polyphenols/mL), but not α-amylase activity. Moreover, when applied with acarbose, HS showed an increased inhibition of α-glucosidase. The α-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 μM. In conclusion, consumption of HS demonstrates potential to beneficially impact mechanisms contributing to blood glucose regulation, and regular consumption should therefore be encouraged.

1. Introduction

Type 2 diabetes (T2D) is characterized by hyperglycaemia and associated with impaired insulin secretion and insulin resistance. Globally, the incidence of T2D is increasing exponentially with 451 million people estimated as diabetic in 2017 and projected to reach 693 million by 2045 [1]. 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 T2D. In particular, reduction of postprandial glycaemia has been highlighted as an effective mechanism to maintain glucose homeostasis and reduce the risk of T2D [2].

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 [2–4]. Several mechanisms have been proposed by which polyphenols may modulate glucose metabolism, such as attenuation of carbohydrate digestion by inhibiting salivary and pancreatic α-amylase enzymes, and α-glucosidases in the small intestinal brush border, as well as inhibition of glucose absorption, stimulation of insulin secretion and protection of pancreatic β-cells against glucotoxicity [2]. Several flavonoids such as naringenin, kaempferol, luteolin, apigenin, (+)-catechin/(−)-epicatechin, daidzein, and epigallocatechin gallate have been reported to inhibit starch digestive enzymes [5]. Further, Zhang et al. [6] has identified four major active phenolic compounds, ellagic acid, cyanidin diglucoside, pelargonidin-3-rutinoside, and catechin present in raspberries as α-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+-dependent SGLT1 and GLUT2 [7]. Anthocyanins are a polyphenol

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subgroup and are widely distributed in edible fruits and vegetables. Their intake has been associated with cardiovascular and metabolic health [8,9]. *In vitro* studies suggest that anthocyanins such as cyanidin and its glucosides can inhibit the activity of amylase and glucosidase [10]. Cyanidin-3-galactoside (IC_{50} 0.05 mM) was found most potent intestinal glucosidase inhibitor whereas cyanidin-3-glucoside (IC_{50} 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 T2D [11].

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 [12]; indeed, a recent meta-analysis of our group has confirmed the consistent effect of HS on systolic blood pressure reduction [13]. 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 [14]. In prediabetic women, the consumption of rosella tea (5 g HS tea with 125 g stevia sweetener) twice a day for 14 days led to a significant decrease in fasting blood glucose (FBG) when compared to the control group [15]. However, a recent acute study could not demonstrate significant differences of postprandial glucose and insulin after consumption of HS drink (7.5 g HS tea in 250 mL water) [16]. The commercially available HS concentrate was selected for the present trial to provide relatively higher amounts of HS.

Given the limited evidence on the topic, the aim of this study was to investigate the inhibitory effect of HS and its main anthocyanins (delphinidin- and cyanidin-sambubiosides) and some of their metabolites on the activity of α-amylase and α-glucosidase enzymes and to establish acute anti-diabetic effects on post-prandial glucose *in vivo* in human volunteers. One objective of the *in vitro* enzyme inhibition experiments was to investigate the potential synergistic effects of HS with the synthetic drug acarbose, which has been shown for some flavonoids and acarbose on α-glucosidase inhibition [17] as well as for cyanidin and its derivatives [10]. The *in vitro* enzyme inhibition assay for α-glucosidase activity was measured through monitoring the kinetics of the reaction, a straightforward and reliable way of measuring enzyme activity, which can be advantageous in particular in highly pigmented samples that might interfere with the measurement.

2. Materials and methods

2.1. Reagents and samples

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). HS concentrate was kindly provided by IBIS Organics, Carlisle, 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). Accu-Chek® Performa Nano including test strips were purchased from a commercial provider (Boots, Leeds, UK). Chemicals such as sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium carbonate, 3,5-Dinitrosalicylic acid (DNS), and sugar standards (D-glucose, D-fructose and D-sucrose), gallic acid, amylose (potato starch), porcine pancreatic amylase (PPA), α-glucosidase (from *Saccharomyces cerevisiae)*, p-nitrophenyl glucopyanoside (pNPG) and Folin reagent were purchased from Sigma-Aldrich (Dorset, 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. UK). Microvette® 200 tubes were obtained from Sarstedt (Leeds, UK), DuoSet Insulin and control were from R&D systems Bio-Techne (Abingdon, UK).

2.2. Characterization of hibiscus samples

2.2.1. Total phenolic content

The total polyphenol content in HS concentrate was determined using the Folin-Ciocalteu method adapted to 96 well plate format [18] with slight modifications. Briefly, 10 μL of diluted sample was mixed with 40 μL of Folin reagent (12.5%) and 150 μL 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 μg/mL) with results expressed as mg/mL GA equivalents.

2.2.2. Anthocyanin analysis

Total anthocyanins in HS concentrate were measured via the pH differential method [19,20]. Briefly, appropriately diluted samples were added to 0.025 M potassium chloride buffer (pH 1) as well as 0.4 M sodium acetate buffer (pH 4.5) and absorbance recorded after 15 min at 520 and 700 nm using a UV/VIS scanning spectrophotometer. Absorbance readings at pH 4.5 were subtracted from pH 1 readings after wavelength correction using the equation: $A = (A_{\lambda vis\text{-}max} - A\ 700)\ pH\ 1.0 - (A_{\lambda vis\text{-}max} - A\ 700)\ pH\ 4.5$. The concentration of total monomeric anthocyanins in the original samples (mg/L) was calculated using the formula $(A \times MW \times DF \times 1000)/(\varepsilon \times 1)$ where MW = molecular weight of delphinidin-3-sambubioside (597.5), DF = dilution factor and ε = molar absorptivity of delphinidin-3-sambubioside (26600).The anthocyanins present in HS were identified via HPLC/MS using the method described by Ifie et al. [21]. HPLC (LC2010 HT) coupled with a 2020 quadrupole mass spectrophotometer (Shimadzu, Kyoto, Japan) fitted with an electrospray ionization source (ESI-MS) with a reverse phase Phenomenex Gemini C_{18} column (4.6 mm × 250 mm, 5 μm) operated in single ion monitoring (SIM) was used in positive mode.

2.2.3. Determination of sugars using HPLC-ELSD

Soluble sugars (glucose, fructose, sucrose) present in HS concentrate were analysed by chromatographic technique as described by [21]. 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 μm, 250 nm × 4.6 mm). The mobile phase was 75% acetonitrile (v/v) with a flow rate of 0.5 mL/min and injection volume of 10 μL. 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.

2.3. In vivo human study

2.3.1. Participant recruitment

The study was approved (MEEC 16–028) by the Research Ethics Committee, University of Leeds, UK. The work has been carried out in accordance with the Declaration of Helsinki (The Code of Ethics of the World Medical Association). The intended sample size of 15 subjects was based on power calculation and similar studies to determine changes in postprandial glucose [22,23]. Healthy participants (6 males; 9 females), meeting the inclusion criteria (aged 20–40 years; average BMI = 24.28 kgm^{-2}, not pregnant or breast-feeding, no taking any medication or supplements) were recruited using advertisements, flyers and personal communications. The participants were asked 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 10 h and no more than 12 h prior to each visit. Anthropometric measurements (height, weight, and waist circumference) were obtained for all subjects during their first visit using a digital scale and measuring tape. All participants completed a health screening questionnaire 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). There was full compliance with study requirements.

2.3.2. Study design

In a randomized controlled trial, participants were invited to attend the human study facilities at the University of Leeds, UK, on three occasions, separated by 2–3 days between each visit. After a baseline blood glucose measurement, volunteers were asked to consume 55 g of bread as well as a drink containing either 30 mL (low) or 50 mL (high) amount of HS concentrate (diluted with water to 300 mL volume) or a sugar matched control drink (300 mL), within a few minutes. Each drink contained the same amount of sugar (25 g sucrose) and provided, together with bread, 50 g available carbohydrates. The detailed composition of the test and control drinks has shown in Table 2. Capillary blood was collected via finger prick at baseline and in regular intervals following drink and bread consumption (0, 15, 30, 45, 60, 90, 120, 150, 180 min) 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. Insulin was determined via immunoassay in duplicates after appropriate dilution.

2.4. Enzyme inhibition in vitro

2.4.1. Inhibition of α-glucosidase activity

Activity of α-glucosidase enzyme was measured according to Zhang et al. [24] with some modifications. Briefly, in wells of a 96 well plate, 100 μL of HS sample (covering a range of 0–400 μg polyphenols/mL) or pure compounds (delphinidin- and cyanidin sambubiosides, protocatechuic acid, gallic acid, chlorogenic acid; ranging from 0 to 100 μM) were incubated with 50 μL of α-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 μL 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 plate reader. 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 α-glucosidase and α-amylase enzymes, was used as a positive control (0–4000 μg/mL).

2.4.2. Inhibition of α-amylase activity

The α-amylase inhibitory effect of HS concentrate was measured using DNS assay according to the procedure prescribed by Nyambe-Silavwe et al. [25]. Briefly, 50 μL of HS sample, mixed with 50 μL phosphate buffer saline (20 mM, pH 6.9) and 200 μL of starch (amylose) solution (2.5 mg/mL), were incubated at 37 °C for 10 min. The reaction was started by the addition of 200 μL porcine pancreatic α-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 at 100 °C for 10 min, transferred to ice to cool down to room temperature and then centrifuged for 5 min. The sample 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 [25]. To the SPE purified sample, 1 mL DNS reagent was added, and the mixture was heated at 100 °C for 10 min. After cooling to room temperature, 250 μL 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: inhibition (%) = [absorbance (control-sample)/control]*100.

Table 1
Compositional analysis of hibiscus concentrate.

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

The results are mean ± SEM of three independent measurements.

2.5. Statistical analysis

Analysis of results from *in vitro* and *in vivo* experiments was conducted using GraphPad Prism; Version 9. The sample size for *in vivo* study was calculated to detect differences of at least one standard deviation of postprandial glycaemic response (1 mmol/L) between the control and HS drinks. According to the calculation, a total of 13 participants would be required for this crossover study for a significance level of 0.05 and a probability of 90%. Linear mixed model with Tukey's adjustment was used to compare the effects of test drinks. The histogram was used for checking the normality of model residuals, and logarithms for non-normally distributed data. Values of $P \leq 0.05$ were considered significant. Incremental blood and insulin values were calculated by subtracting baseline values from all subsequent time points and AUC was determined. In addition, peak plasma insulin and glucose values were identified. *In vitro* experiments were conducted at least in thrice in duplicates. IC_{50} for enzyme assays were estimated by non-linear regression analysis using the GraphPad Prism software. All results *(in vitro* and *in vivo*) are expressed as mean ± standard error mean or confidence interval (CI).

3. Results

3.1. 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 (Supplementary Fig. 1). Total anthocyanin and polyphenol content are shown in Table 1. 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 1). However, given that the sugar content was below <1g drinkable portion, it was not considered to have an effect on postprandial glucose response.

3.2. Human study: glycaemic response

Fifteen volunteers who met the edibility criteria were randomized to test drinks and completed the study. The postprandial changes in blood glucose after consuming control/HS drink with 50 g available carbohydrates are shown in Fig. 1. 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 (Fig. 1A). 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 (Fig. 1B). 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 (Fig. 1C).

Table 2
Characteristics/composition of test meals/drinks.

type of drink	fructose (g)	glucose (g)	sucrose (g)	total intrinsic sugars (g)	sugar from bread (g)	added sucrose (g)	total sugars (g)	total PP (mg)	total ACNs (mg)
Control	0	0	0	0	25	25	50	0	0
Low dose	0.20	0.24	0.08	0.52	25	25	50.52	230	132
High dose	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.

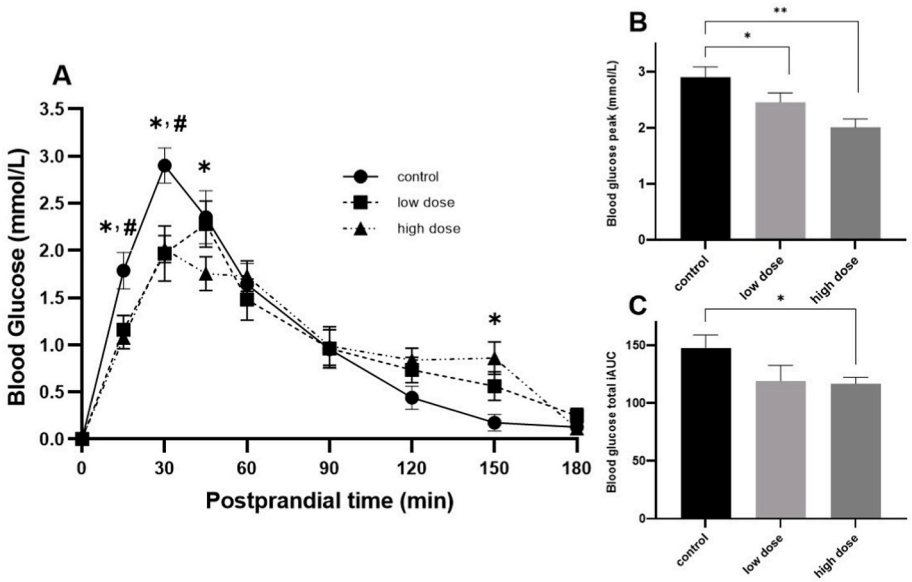

Fig. 1. Incremental blood glucose response over 180 min following consumption of control drink (●) and either low (■) or high dose hibiscus (▲) sugar matched drinks (A); as well as peak plasma glucose (B) and total iAUC (C). The data represent means with SEM of 15 participants. Post hoc analysis of time-point 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.

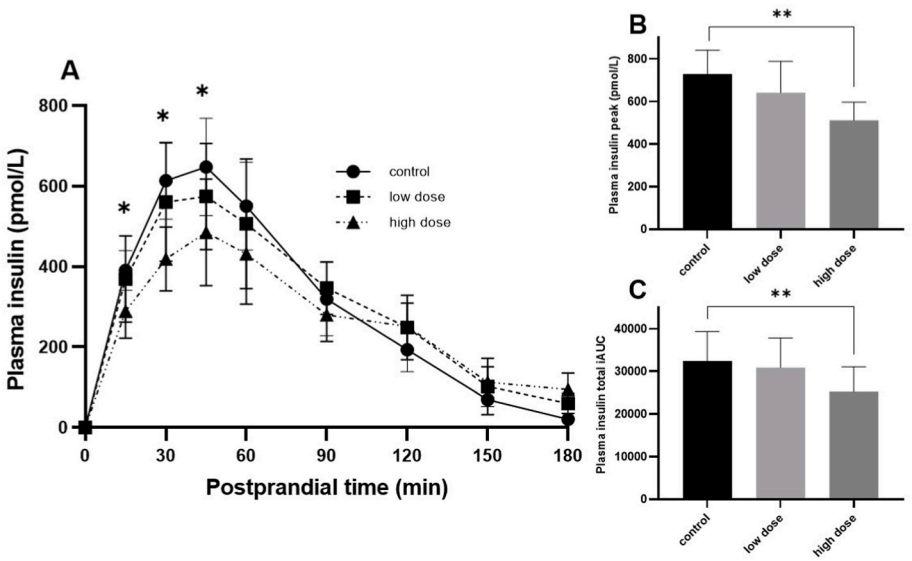

Fig. 2. Incremental plasma insulin response over 180 min following consumption of control drink (●) and either low (■) or high dose hibiscus (▲) sugar matched drinks (A). (B) indicates plasma peak insulin concentrations and (C) total iAUC. Data are mean with SEM of 9 participants. Post hoc analysis of 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.

Postprandial plasma insulin data, shown in Fig. 2, 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 (Fig. 2A). This was also reflected in plasma peak insulin (Fig. 2B) and total insulin iAUC (0–120 min) (Fig. 2C) which were both significantly lower following high HS intake ($P = 0.048$, $P = 0.010$) but not after low HS dose ($P = 0.591$, $P = 0.099$).

3.3. Inhibition of α-glucosidase and α-amylase in vitro

Inhibition of α-glucosidase was determined by the rate of release of p-nitrophenol (yellow colour compound) from p-nitrophenyl glucopyranoside at 37 ^{0}C. As shown in Fig. 3A, acarbose, a synthetic inhibitor of

α-glucosidase which was used as positive control, dose-dependently inhibited enzyme activity with IC_{50} 619 μg/mL at 95% CI (556.4–676.0).

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 (Fig. 3C). In addition, wavelength scans (350–600 nm) confirmed the does-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 Fig. 3D. As shown in Fig. 3B, HS concentrate also inhibited α-glucosidase activity in a dose-dependent manner with IC_{50} 120.9 μg polyphenols/mL at 95% CI (113.8–128.4). Similarly to acarbose, the absorbance readings demonstrate linearity over time (Fig. 3E; $r > 0.99$) and clearly document product formation inhibition through wavelength scan (Fig. 3F) in these samples which were corrected for their individual background absorbance.

In order to identify the contribution of individual anthocyanins to α-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 μM (Supplementary Fig. 2); and only a weak inhibition (<10%) was observed for the phenolic acids at 100 μM (Supplementary Fig. 3).

In addition, we aimed to establish whether HS and acarbose might act synergistically on α-glucosidase. Therefore, the assay was performed in the presence of both, acarbose and different concentrations of HS. As results in Fig. 4A demonstrate, the dose-dependent glucosidase inhibitory effect of acarbose was enhanced by increasing amounts of HS in the reaction. As shown in Fig. 4B and C, the addition of HS (191.5 and 95.7 μg/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 α-amylase enzyme (IC_{50} 37.6 μg/mL), however, neither HS concentrate (up to 400 μg polyphenols/mL) nor individual anthocyanins or their metabolites (up to 100 μM) had an effect on enzyme activity (data not shown).

4. 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 α-amylase and α-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. 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 resulted 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 [26]. 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 [27]. As well, the acute consumption of HS extract (providing 311 mg polyphenols and 150 mg anthocyanins) has shown no significant effects on postprandial glycaemic response [16]. In contrast, the present trial provided 2 and 3 times the amount of polyphenols (as compared to the HS tea study) 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 plasma postprandial glucose response, in particular in the early postprandial phase in humans [22,23,28]. 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 [29].

The glycaemic 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 [30]. Cyanidin-3-rutinoside, which is mainly found in blackcurrants [31], but not in lingonberries [32], has indeed demonstrated strong *in vitro* glucosidase inhibition and *in vivo* attenuation of sucrose-induced hyperglycaemia in rats [33]. 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 [23], but berries had no effect on glucose response when consumed with starch-rich pancakes [34]. Similarly, Torronen et al. [22] 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 α-glucosidase than α-amylase [35]. Cleavage of disaccharides such as sucrose is facilitated by intestinal glucosidases. Therefore, the inhibition of α-glucosidase (sucrase activity) by berries in the case of sucrose and poor inhibition of α-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 vitro* findings.

Currently used antihyperglycaemic 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 [36–38]. Importantly, daily intake of acarbose for 3 years reduced the risk for developing type 2 diabetes by 6% compared to control [39]. 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 α-amylase and α-glucosidase inhibitors, as an alternative to acarbose [5]. In particular, anthocyanin-rich berries such as raspberries, strawberries, blueberries and black currant have demonstrated inhibitory properties towards

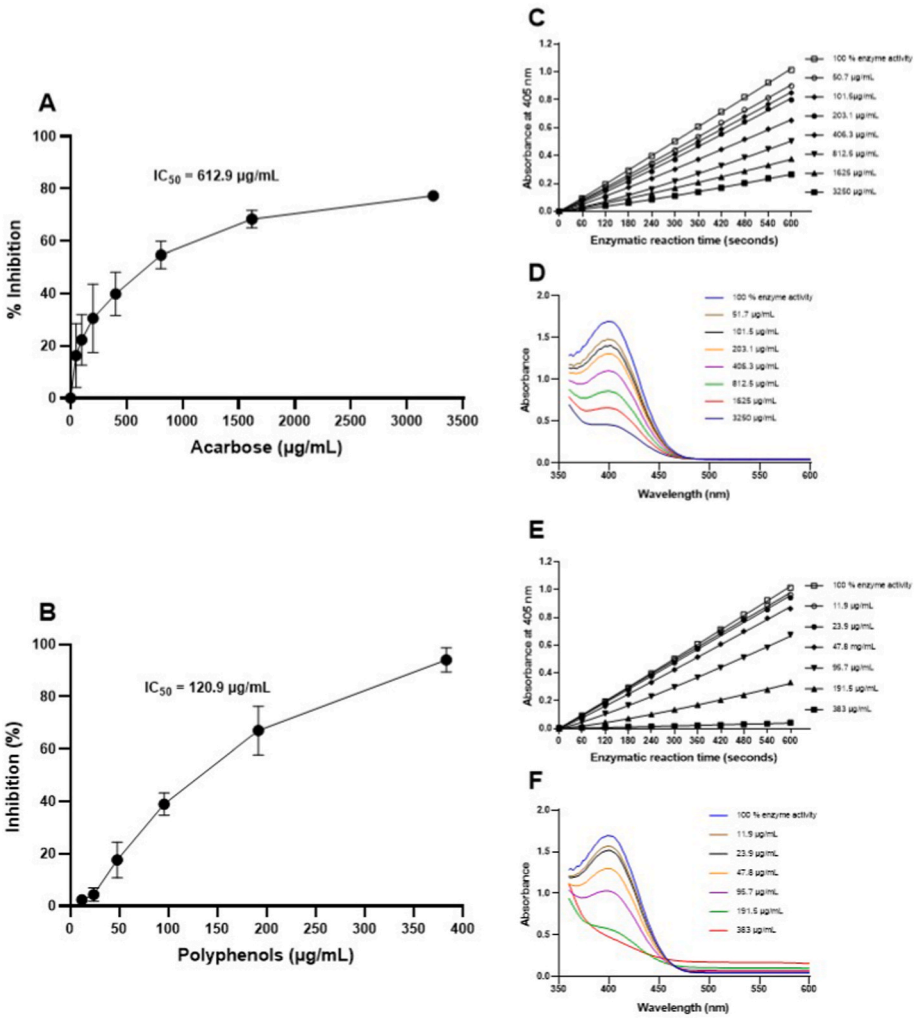

Fig. 3. Dose dependent inhibition of α-glucosidase enzyme by acarbose (A) ranging from 0 to 4000 µg/mL and HS extract (B) ranging from 0 to 400 µg polyphenols/mL. The results are expressed as mean with SEM of three independent measurements performed in duplicate. Kinetic measurement of α-glucosidase 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.

α-amylase and α-glucosidase *in vitro* and *in vivo* [40,41]. The ability of berries to inhibit α-glucosidase was related to their anthocyanin content as described by [41]. Anthocyanins such as cyanidin-3-rutinoside and cyanidin-3-galactoside (IC_{50} 0.05 mM against intestinal sucrase) have been reported as *in vitro* α-glucosidase inhibitors [42,43]. Cyanidin-3-rutinoside is one of the major anthocyanins in blackcurrants, and it showed α-glucosidase inhibitory activity (IC_{50} 19.7 µM) comparable with voglibose (IC_{50} 23.4 µM) [43].

The present study investigated the effect of HS and its anthocyanins for intestinal α-glucosidase and pancreatic α-amylase inhibitory activities. Current *in vitro* data show that HS concentrate is a potent α-glucosidase inhibitor (IC_{50} 120.9 µg polyphenols/mL), lower than the synthetic inhibitor acarbose (619 µg/mL). Our findings are in line with the literature indicating the dose dependent inhibition of α-glucosidase by different varieties of HS. The IC_{50} values of the dark and light red varieties were 165 and 133 µg polyphenols/mL, respectively [21]. Enzyme inhibitory properties of cold and hot aqueous preparations of HS, were reported with IC_{50} of 627 µg/mL and 723 µg/mL, respectively [44]. In contrast, α-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 µM). Similarly, negligible inhibition (<10%) of human salivary α-amylase by HS extract was previously reported [21]. The results of the present trial indicate that pure anthocyanins and their metabolites exert very weak (<10%) inhibition of α-glucosidase up to a concentration of 100 µM. In addition to anthocyanins, there are other polyphenolic compounds in HS, including hibiscus acid, caffeic acid, quercetin, and rutin [21]. Although not investigated in the present study, these compounds have shown inhibitory effects against carbohydrate digesting enzymes [25,45].

This result apparently contradicts earlier published data on anthocyanins; showing α-glucosidase inhibition by cyanidin sambubioside (IC_{50} 543 µM) and delphinidin sambubioside (756 µM), albeit high IC_{50} 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 α-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 [42]. Cyanidin-based anthocyanins have been determined for their combined effect with acarbose against α-glucosidase and α-amylase [10,42,46]. Recent review on natural compounds and pharmaceutical agents used for the treatment and management of T2D highlighted synergistic interactions between synthetic drugs and natural inhibitors (polyphenols) to provide with a better approach to minimizing the side effects of synthetic inhibitors [47].

Besides polyphenols/anthocyanins, other properties of foods/beverages, such as pH, have been reported as contributors to lower activity of carbohydrate digesting enzymes. Freitas et al. [48] have 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

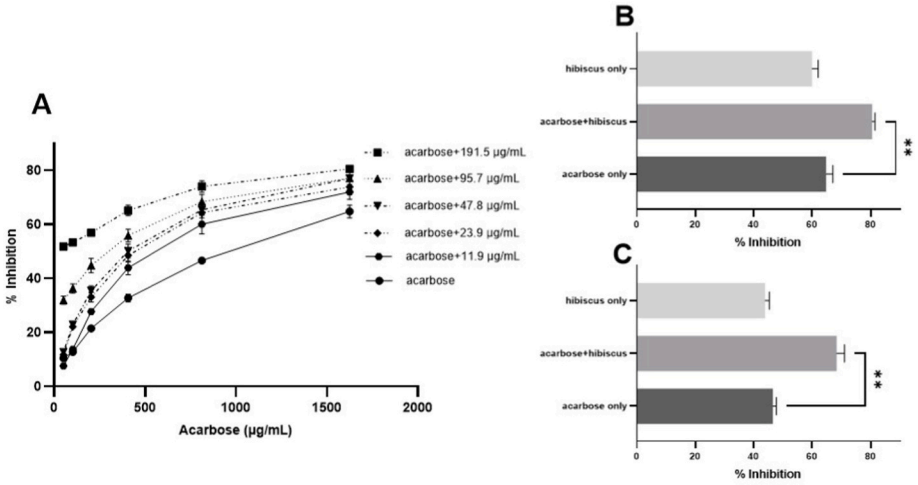

Fig. 4. Effect of different concentrations of hibiscus polyphenols and acarbose on α-glucosidase inhibition (A). The % inhibition at selected concentrations; 191.5 μg polyphenols/mL HS extract +1625 μg/m/L acarbose (B) and 95.7 μg polyphenols/mL HS extract + 812.5 μg/mL acarbose (C) is increased. Data are mean with SEM of three experiments performed in duplicate. * indicates significant difference of hibiscus-acarbose combination versus acarbose ($P < 0.05$, *t*-test).

the results of an *in vivo* trial [49] where only lemon juice in contrast to water or tea (alongside bread consumption), showed a reduced (and delayed) peak blood glucose concentration [49]. 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 α-amylase activity indirectly as there was no direct effect on pancreatic α-amylase activity *in vitro*. However, *in vitro* α-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 α-amylase and intestinal a-glucosidase, respectively, have contributed to lower the postprandial glucose response *in vivo*. Further studies are needed to better understand the contribution and relevance of pH in contrast and/or in combination with bioactive effects in the regulation of glucose metabolism.

Despite the fact that extrapolating *in vitro* and *in vivo* results can be challenging, it is assumed that anthocyanins reach high concentrations (i.e. in the μM range) in the gastrointestinal tract shortly after consumption. In the present study, *in vitro* inhibition experiments using HS covered a range of concentrations (12–400 μg polyphenols/mL) with an IC_{50} value of 120.9 μg/mL, as shown in Fig. 3. Considering further dilution through intestinal fluids, the *in vivo* concentrations should be 3 times more than *in vitro* [7]. The HS intervention (high dose) provided 383 mg total polyphenols (1.27 mg/mL) which was 10.5 times higher than the IC_{50} value; it can therefore be assumed that the *in vitro* concentrations that were used in the current study, were in an appropriate range.

The lower concentrations of individual anthocyanins (<100 μM) tested for *in vitro* α-glucosidase inhibition compared to those present in HS drink (>100 μM) 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 [13]. Therefore, further studies investigating chronic effects of HS intake on markers of glycaemia are urgently needed.

5. 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 α-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 α-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.

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Author contributions

SZ, CB, LM – conceptualization; data curation; SZ, CB - formal analysis; SZ – investigation; SZ, CB – methodology; SZ - project administration; CB, LM – resources; CB, LM – supervision; SZ – validation; visualization; SZ - original draft writing; CB, LM - review and editing. All authors read and approved the final draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.hnm.2022.200164.

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