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In vitro α-glucosidase inhibition by honeybush (*Cyclopia genistoides*) food ingredient extract—potential for dose reduction of acarbose through synergism†

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Extracts of *Cyclopia* species are used as food ingredients. *In vitro* α-glucosidase (AG) inhibition by ultrafiltered *C. genistoides* extract, fractions enriched in xanthones (XEF) and benzophenones (BEF), as well as mangiferin, isomangiferin, 3-β-D-glucopyranosyliriflophenone (I3G) and 3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone (IDG) was determined with acarbose as positive control. XEF was more potent than the extract and BEF (IC_{50} = 43.3, 95.5 and 205.7 μg mL^{-1}, respectively). Compounds demonstrated potency in the descending order: acarbose (IC_{50} = 44.3 μM) > mangiferin (102.2 μM) > isomangiferin (119.8 μM) > I3G (237.5 μM) > IDG (299.4 μM). The combination index (CI) was used to determine synergism (CI < 0.7) as demonstrated for combinations of acarbose with XEF, BEF or the respective compounds at 50% and 75% effect levels. The greatest potential acarbose dose reductions (>six-fold) across all effect levels were calculated for combinations of acarbose with mangiferin or isomangiferin, explaining the greater acarbose dose reduction potential of XEF *vs.* BEF. The effect of batch-to-batch variation (n = 10) of raw plant material on AG inhibition was quantified at a fixed concentration (160 μg mL^{-1}). XEFs (xanthone content = 223–481 g kg^{-1}) achieved AG inhibition of 63–72%, whereas BEFs (benzophenone content = 114–251 g kg^{-1}) achieved AG inhibition of 26–34%, with weak linear correlation (R^2 < 0.43) between target compound content of the fractions and their achieved AG inhibition. Thus, extract fractions of *C. genistoides*, enriched in xanthones and benzophenones, show potential in reducing the effective dose of acarbose required to prevent postprandial hyperglycaemia.

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Introduction

Polyphenol-rich extracts are increasingly finding application in functional foods and beverages, driven by the rise in health awareness of consumers.[1] Mounting scientific evidence of the role of polyphenols in the management of chronic metabolic and lifestyle-related diseases such as type 2 diabetes mellitus,[2] and the strong association between the diet and diabetes, underpin the development of nutraceuticals or functional food ingredients with specific anti-diabetic or anti-obesity bioactivity.[3] Diabetes, pre-diabetes and impaired glucose tolerance, along with the associated rise in obesity and the metabolic syndrome, have become major health issues across the globe. The International Diabetes Federation has estimated that, as of 2017, approximately 425 million adults (aged 20–79) were living with diabetes.[4] This was projected to escalate to 629 million by the year 2045. Abnormally high levels of blood glucose can eventually lead to chronic complications of the skin, eyes, heart, kidneys and the vascular and peripheral nervous systems.[5,6]

Pharmacological treatment with oral anti-diabetic agents is prescribed when simple lifestyle and dietary interventions alone do not provide adequate blood glucose control.[7] Amongst these, intestinal α-glucosidase inhibitors (AGIs) have been cited as most effective in terms of long-term blood glucose control and regulation of insulin homeostasis.[8] Acarbose, the most widely used commercial AGI, is sometimes associated with discouraging dose-related gastrointestinal side effects, related to its strong affinity for the enzyme binding site (approximately 105 times that of dietary oligosaccharides), which results in an increased load of undigested carbohydrates making its way to the colon where fermentation by gut flora takes place.[9] The search for alternatives includes plant extracts

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or purified phytochemicals with α-glucosidase inhibitory activity.[7,10–12] The glycosylated xanthones, mangiferin and isomangiferin, and benzophenones (3-β-D-glucopyranosyliriflophenone, I3G, and 3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone, IDG), found in significant amounts in *Cyclopia genistoides*, have also been confirmed as active inhibitors of mammalian α-glucosidase.[13,14] These findings laid the foundation for the optimisation of scalable extraction[14] and ultrafiltration[15] unit operations, as well as a protocol for the preparation of xanthone- and benzophenone-enriched fractions,[16] using green (unoxidised/'unfermented') *C. genistoides* plant material as starting material. The aim is to develop a food ingredient that can be used in a beverage for consumption before or during a meal to curb rapid breakdown of carbohydrates. *Cyclopia genistoides* is one of the *Cyclopia* species that is used for production of the herbal tea, known as honeybush tea.[17] A beverage such as a ready-to-drink iced tea would therefore present consumers with a product option that could deliver the food ingredient extract or crude polyphenol-enriched fraction from *C. genistoides* as part of the diet.

The reported inhibitory effects of natural AGIs are generally less potent than commercial inhibitors such as acarbose, which often serves as the positive control in bioactivity testing.[12] Some recent studies have reported synergistic α-glucosidase inhibition by combinations of acarbose and botanical extracts or plant phenolics.[18–21] Of particular interest is a study showing that a combination of acarbose and *Oroxylum indicum* seed extracts not only resulted in a synergistic effect *in vitro*, but also resulted in enhanced efficacy of acarbose *in vivo*.[22]

Prompted by long-standing disparity in scientific literature regarding the definition and proper evaluation of synergism, Chou & Talalay[23,24] introduced the concept of the combination index (CI), which provides a definition for synergism, antagonism and additive effects in combination therapy based on the law of mass action. At the most basic level, synergism/antagonism refers to a combined effect that is more/less than additive. The Chou–Talalay method offers an advantage over the traditional isobologram method of assessing synergism in that it provides numerically indexed conclusions, *i.e.* a quantitative measure of synergism, in the form of the CI.[25] Other benefits of the CI method include its simplicity, flexibility (mechanism- and unit-independence) and economy (requires small number of data points).[26] It is already known that the inhibitory activity of AGIs varies depending on the origin of the enzyme.[27] Potent inhibitors of non-mammalian α-glucosidase will often prove to be poor inhibitors of mammalian α-glucosidase under the same experimental conditions.[28,29]

The present study aimed to determine whether a multi-step enrichment protocol for the development of a xanthone-enriched fraction (XEF) and a benzophenone-enriched fraction (BEF) of *C. genistoides* enhanced α-glucosidase inhibition. Furthermore, combinations of acarbose and respectively, the fractions and the four major phenolic compounds found in *C. genistoides* (mangiferin, isomangiferin, IDG and I3G), were investigated for *in vitro* synergistic inhibitory activity against α-glucosidase, using the Chou–Talalay method. It was also deemed imperative to delineate the effect of variable phenolic content of the fractions on α-glucosidase inhibitory activity and potential for acarbose dose reduction, as large batch-to-batch variation in the composition of source material is highly likely. Mammalian α-glucosidase was used instead of the frequently used commercial yeast α-glucosidase preparations, because of the intended anti-diabetic application of the final product.

Materials and methods

Chemicals and reagents

Authentic reference standards (purity >95%) for the enzyme assay and high-performance liquid chromatography (HPLC) quantification were obtained from Sigma-Aldrich (St Louis, MO, USA; mangiferin), and Phytolab (Vestenbergsreuth, Germany; I3G, isomangiferin). IDG (purity >95%) was previously isolated from *C. genistoides* in our laboratory.[13] Acarbose, rat intestinal acetone powder and 7-*O*-α-D-glucopyranosyl-4-methylumbelliferone (MUG) were supplied by Sigma-Aldrich. HPLC gradient grade 'far UV' acetonitrile was supplied by Merck (Darmstadt, Germany). All other reagents, except ethanol (Servochem, Cape Town, South Africa), were analytical grade and supplied by Sigma-Aldrich or Merck. Deionised water, prepared using an Elix Advantage 5 (Merck) water purification system, was further purified to HPLC grade using a Milli-Q Reference A+ (Merck) water purification system.

Cyclopia genistoides extract, ultrafiltration products and enriched fractions

Extraction of green *C. genistoides* plant material and ultrafiltration of the extract (23 L) have been previously described.[14,15] Briefly, the plant material was extracted in a 1 : 10 solid : solvent ratio (m v^{-1}) for 30 min at 70 °C, using a 40% ethanol-water mixture (v v^{-1}). This extract served as the initial feed (IF) for tangential ultrafiltration through a 10 kDa regenerated cellulose membrane to obtain the ultrafiltered extract ($UCGE_0$) and retentate (R).[15] All were tested for α-glucosidase inhibition in the present study (Fig. 1). Two fractions, respectively enriched in xanthones (XEF_0) and benzophenones (BEF_0), were obtained for α-glucosidase inhibition testing by combining an equal mass of triplicate fractions (XEF A–C; BEF A–C), previously produced by macroporous adsorbent resin chromatography (MARC).[16] In order to test the effect of natural batch-to-batch variation in the composition of the plant material on α-glucosidase inhibition by enriched *C. genistoides* fractions, $XEF_{1–10}$ and $BEF_{1–10}$, produced in triplicate from 10 different batches, were prepared. Their preparation by MARC is described in Miller *et al.*[16] The different batches of plant material were harvested during June 2018 from two plantations, situated at Toekomst farm (Bredasdorp, South Africa; GPS coordinates: −34.54340, 19.87983) (n = 9) and Tygerhoek Research farm (Riviersonderend, South Africa; GPS coordinates: −34.14469, 19.90169) (n = 1), respectively.

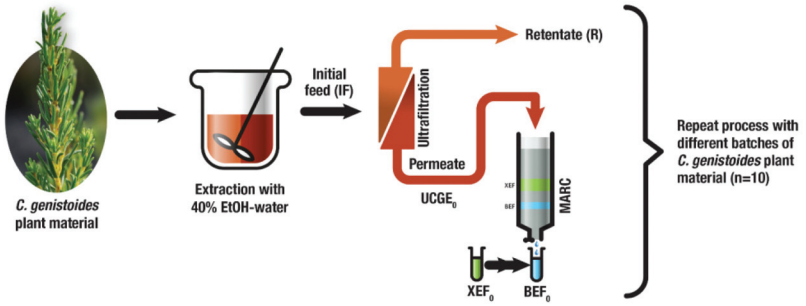

Fig. 1 Schematics of enrichment processes for xanthones and benzophenones in *Cyclopia genistoides* extract, serving as test samples for α-glucosidase inhibition in the present study (BEF = benzophenone-enriched fraction; MARC = macroporous adsorbent resin chromatography; $UCGE_0$ = ultrafiltered *Cyclopia genistoides* extract; XEF = xanthone-enriched fraction).

α-Glucosidase inhibition

Determination of α-glucosidase inhibition was carried out according to a fluorimetric method adapted from Bosman *et al.*[14] An extract of rat intestinal acetone powder containing α-glucosidase was prepared by suspending *ca.* 1050 mg of powder in 30 mL of cold KH_2PO_4 buffer (200 mM KH_2PO_4, pH 6.8 with KOH), followed by sonication on ice. The crude mixture was centrifuged at 10 000*g* for 15 min and the supernatant was retrieved and filtered (0.45 μm, 33 mm Millex HV PVDF filter membranes, Merck). The filtered supernatant was used as an enzyme mixture after dilution to a standardised concentration based on activity testing. Activity determination of the enzyme mixture was performed daily prior to each set of experiments, using the same procedure as for the inhibition assays, but with H_2O as sample control and varying dilutions of the enzyme mixture. Fluorescence measurements, performed on a BioTek SynergyHT microplate reader (BioTek Instruments, Winooski, VT, USA), were used to determine the correct concentration for optimal enzyme activity estimated as an FL-value of 50 000 (λ_{EX}: 360 nm; λ_{EM}: 460 nm), 20 min after addition of the substrate (MUG).

The following test procedure was employed: 80 μL of the assay control (H_2O), positive control (acarbose) or test sample, diluted with 200 mM KH_2PO_4 buffer (pH 6.8) to the selected concentration, was added to 65 μL of a 200 mM KH_2PO_4 buffer (pH 6.8) and 65 μL of the pre-determined dilution of the enzyme mixture in 96-well, black microplates with clear, flat bottoms (Greiner Bio-One GmbH, Kremsmünster, Austria). After pre-incubation at 37 °C for 15 min, 40 μL of a 1.2 mM MUG solution was dispensed at t = 0 min. Fluorescence (λ_{EX}: 360 nm; λ_{EM}: 460 nm) was monitored over 30 min, and the nett fluorescence (Nett FL), remaining enzyme activity (%) and α-glucosidase inhibition (%) were calculated using the following equations:

$$\text{Nett FL} = FL_{30} - FL_0 \quad (1)$$

$$\text{Remaining enzyme activity}(\%) = 100 \times \left(\frac{\text{Nett FL}_s}{\text{Nett FL}_{ac}}\right) \quad (2)$$

$$\text{Enzyme inhibition}\ (\%) = 100 - \text{remaining enzyme activity} \quad (3)$$

FL_0 and FL_{30} represent the fluorescence intensity measured at 0 and 30 min, respectively, and Nett FL_s and Nett FL_{ac} refer to the Nett FL calculated for the sample and assay control, respectively. For determination of IC_{50} values a concentration range was employed. BEFs and XEFs prepared from the different batches of *C. genistoides* plant material were tested at a fixed reaction concentration (160 μg mL^{-1}). All sample concentrations were analysed in triplicate.

Assessment of synergistic α-glucosidase inhibition. Synergistic interaction between the various AGIs under investigation (IF, R, $UCGE_0$, XEF_0, BEF_0, isomangiferin, mangiferin, I3G, IDG and acarbose) was evaluated according to the CI method.[30] The AGIs were combined in a 1 : 1 ratio based on μg mL^{-1} concentration in the reaction volume. The same test procedure described in the previous section was used, except that 40 μL of each AGI in the combination was added to the reaction volume, *i.e.* 80 μL of the combination under investigation was added. The dose-effect data of the combinations were analysed using freely available software (CompuSyn Version 1.0; http://www.combosyn.com/index.html).[30] α-Glucosidase inhibition values (%) were converted to F_a (effect level) values (0–1) for CompuSyn analyses by dividing by 100. CI is represented by the following equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2} \quad (4)$$

where $(D)_1$ and $(D)_2$ are the doses of inhibitors that produce a specified level of inhibition in the combination system, and $(D_x)_1$ and $(D_x)_2$ are the doses of these inhibitors that would result in the same effect when used alone. CI values were calculated at 25, 50 and 75% inhibition, *i.e.* at effect levels 0.25, 0.5 and 0.75, representing moderate enzyme inhibition. The combined inhibition was classified as synergistic (CI < 0.9), additive (CI = 0.9–1.1), or antagonistic (CI > 1.1). The dose reduction indices (DRIs) for both agents in a given combination, derived from CI (eqn (4)), were included as part of the standard CompuSyn data output. This represents the theoretical *x*-fold dose reduction of each inhibitor in a synergistic

combination that may be achieved at a given effect level relative to the same inhibitor when used alone.[30]

High-performance liquid chromatography with diode-array detection (HPLC-DAD)

HPLC-DAD analysis of samples was performed using a validated method, developed specifically for green *C. genistoides*.[31] The instrument consists of an Agilent 1200 series system with an in-line degasser, autosampler, column thermostat, quaternary pump and diode array detector (Agilent Technologies Inc., Santa Clara, CA, USA). Separation was achieved on a Kinetex column (150 × 4.6 mm ID, 2.6 μm dp; Phenomenex, Torrance, CA, USA) maintained at 30 °C, with the mobile phase consisting of (A) 1% aqueous formic acid (v v^{-1}), (B) methanol and (C) acetonitrile, at a flow rate of 1.0 mL min^{-1}. Multi-linear gradient elution was carried out as follows: 0 min (95.0% A, 2.5% B, 2.5% C), 5 min (95.0% A, 2.5% B, 2.5% C), 45 min (75% A, 12.5% B, 12.5% C), 55 min (50% A, 25.0% B, 25.0% C), 56 min (50% A, 25.0% B, 25.0% C), 57 min (95.0% A, 2.5% B, 2.5% C), 65 min (95.0% A, 2.5% B, 2.5% C). UV-Vis spectra were recorded at 200–700 nm, with selective wavelength monitoring at 288 nm (benzophenones) and 320 nm (xanthones). The quantification of the compounds was based on six-point calibration curves, spanning expected concentration ranges. IDG and isomangiferin were quantified using response factors *vs.* I3G and mangiferin, respectively. Ascorbic acid was added to samples prior to analysis to prevent oxidation of compounds. After mixing, the samples and standard mixtures were filtered using 0.45 μm Millex-HV syringe filters (Merck).

Statistical analysis

Half-maximal inhibitory concentrations (IC_{50}) were calculated by non-linear regression analysis of the dose-effect data using GraphPad Prism (Version 8.2.1; GraphPad Software, San Diego, CA, USA). The four-parameter variable slope regression model was used, with the bottom and top values for the remaining α-glucosidase activity constrained between constant values of 0 and 100, respectively. IC_{50} values are represented as means with 95% confidence intervals. Regression analysis of α-glucosidase inhibition values against xanthone and benzophenone contents of the enriched fractions was performed using Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

Results and discussion

Effects of enrichment processes on α-glucosidase inhibitory activity

Tangential flow ultrafiltration and MARC represent two consecutive unit operations in an eco-friendly, scalable enrichment process, with the ultrafiltered *C. genistoides* extract ($UCGE_0$) serving as the starting material for the production of xanthone- and benzophenone-enriched fractions (XEF_0, BEF_0) by MARC (Fig. 1). In the present study, these samples, as well as the initial feed (IF prior to ultrafiltration) and the retentate (R), were investigated in terms of their inhibitory activity against mammalian α-glucosidase. Dose–response curves (Fig. 2a) and their derived IC_{50} values (mean with 95% confidence interval) (Table 1) indicate that $UCGE_0$ was more potent (IC_{50} = 95.5 μg mL^{-1}) than the starting material, IF (IC_{50} = 115.3 μg mL^{-1}), and R, the ultrafiltration by-product containing retained or "rejected" material (IC_{50} = 153.3 μg mL^{-1}). Ultrafiltration was previously shown to achieve good enrichment in xanthones and benzophenones relative to IF.[16] Despite R being the "non-enriched" fraction of the ultrafiltration process, it still displayed appreciable inhibitory activity. Substantial amounts of mangiferin, isomangiferin, I3G and IDG, all reported as active AGIs,[13,14] were still present in R (Table 1), partially explaining its activity.

The dose-effect curves for the phenolic compounds and acarbose are depicted in Fig. 2b. Mean IC_{50} values for the phenolic compounds of interest (Table 1) indicate a descending order of potency (mangiferin > isomangiferin > I3G > IDG). All compounds were less potent than acarbose (IC_{50} = 44.3 μM =

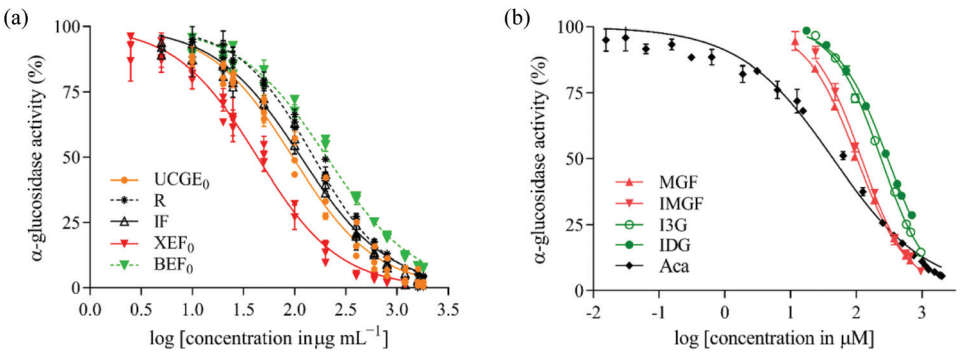

Fig. 2 (a) Logarithmic dose–response curves for five rat intestinal α-glucosidase inhibitors derived from *Cyclopia genistoides* extract: ultrafiltered *C. genistoides* extract ($UCGE_0$), ultrafiltration retentate (R) and initial feed (IF), and xanthone-enriched fraction (XEF_0) and benzophenone-enriched fraction (BEF_0) produced by macroporous adsorbent resin chromatography. (b) Logarithmic dose–response curves for phenolic compounds (3-β-D-glucopyranosyliriflophenone, I3G; 3-β-D-glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone, IDG; mangiferin, MGF; isomangiferin, IMGF) tested for inhibitory activity against mammalian α-glucosidase with acarbose (Aca) as positive control. Data are presented as mean ± standard deviation (n = 3).

Table 1 Half-maximal inhibitory concentrations (IC_{50}) against rat intestinal α-glucosidase for different *Cyclopia genistoides* extract products, and for single compounds including acarbose (positive control)

	Content[a] (g kg^{-1})					
Sample	MGF[b]	IMGF[c]	I3G[d]	IDG[e]	IC_{50}[f] (μg mL^{-1})	95% confidence interval
$UCGE_0$[g]	118.0	33.6	14.8	11.8	95.5	90.5–101.1
Initial feed (IF)[h]	98.2	27.4	11.9	9.3	115.3	109.3–121.5
Retentate (*R*)	61.1	16.7	6.8	5.3	153.3	147.0–159.8
XEF_0[i]	370.5	110.6	33.3	—	43.3	41.1–45.6
BEF_0[j]	6.2	0.3	52.2	70.4	205.7	197.2–214.4
MGF	>95%	—	—	—	43.1[k]	41.4–44.9
IMGF	—	>95%	—	—	50.5[l]	48.2–53.0
I3G	—	—	>95%	—	96.9[m]	93.8–100.1
IDG	—	—	—	>95%	171.1[n]	167.0–175.3
Acarbose	—	—	—	—	28.6[o]	25.0–31.7

[a] Ultrafiltration fractions ($UCGE_0$, IF, R) previously analysed by HPLC-DAD (Miller *et al.*, 2020[16]). [b] Mangiferin. [c] Isomangiferin. [d] 3-β-D-Glucopyranosyliriflophenone. [e] 3-β-D-Glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone. [f] Mean half-maximal inhibitory concentration. [g] Ultrafiltered *Cyclopia genistoides* extract. [h] 40% aqueous ethanol extract of green *C. genistoides*. [i] Reference xanthone-enriched fraction. [j] Reference benzophenone-enriched fraction. [k] Equivalent to 102.2 μM. [l] Equivalent to 119.8 μM. [m] Equivalent to 237.5 μM. [n] Equivalent to 299.4 μM. [o] Equivalent to 44.3 μM.

28.6 μg mL^{-1}), which was still partially effective (<20% inhibition) even at low concentrations (<3 μM). Previously reported inhibitor constants for acarbose (K_i = 0.059 μM)[32] and mangiferin (K_i = 166 μM)[33] against *in vitro* rat intestinal α-glucosidase activity support these findings, as K_i reflects the binding affinity of the inhibitor to the enzyme.[34]

Beelders *et al.*,[13] using fixed concentrations to compare compounds, reported that the additional *O*-glucopyranosyl moiety of IDG at C-4 could explain its weaker inhibition of mammalian α-glucosidase compared with I3G. Similarly, Feng *et al.*[35] noted that another iriflophenone diglucoside, 3,5-β-D-glucopyranosyliriflophenone, was also a weaker inhibitor of α-glucosidase than I3G.

The relative inhibitory activity determined for mangiferin and isomangiferin in the present study (IC_{50} = 102.2 μM and 119.8 μM, respectively) corresponds with previous data,[14] which compared inhibitory activity of mangiferin and isomangiferin against mammalian α-glucosidase at fixed concentrations. Both compounds previously achieved roughly 50% inhibition at 100 μM, with mangiferin slightly more potent than isomangiferin.[14] Of the two enriched fractions of *C. genistoides*, XEF_0 was the more potent AGI, with a mean IC_{50} = 43.3 μg mL^{-1} compared to a mean IC_{50} = 205.7 μg mL^{-1} for BEF_0, ranking thus as the least potent of the products under investigation from the enrichment process (Fig. 1). Acarbose (IC_{50} = 28.6 μg mL^{-1}) was more potent than XEF_0. There was a strong inverse relationship between the xanthone content of the various samples collected during the multi-step enrichment process (IF, R, $UCGE_0$, XEF_0 and BEF_0) and their IC_{50} values for α-glucosidase inhibition. In contrast, the fraction with the highest benzophenone content (BEF_0) had the highest IC_{50}. This can be related to IC_{50} values for the single compounds (Table 1), which clearly demonstrate the superior inhibitory activity of the xanthones when compared to the benzophenones.

Effect of quantitative phenolic variation on α-glucosidase inhibition

BEFs and XEFs, previously prepared from different batches (n = 10) of *C. genistoides* plant material to accommodate natural batch-to-batch variation in the composition of the raw material that could be expected in commercial production of a food ingredient product,[16] were tested in the present study for α-glucosidase inhibitory activity at a fixed concentration (160 μg mL^{-1}). XEFs showed higher overall efficacy than BEFs. XEFs, with xanthone contents ranging from 222.57 to 480.8 g kg^{-1}, achieved α-glucosidase inhibition ranging from 63 to 72% (Fig. 3a). BEFs, with benzophenone contents ranging from 113.84 to 251.21 g kg^{-1}, achieved α-glucosidase inhibition ranging between 26 and 34% (Fig. 3b). There was a weak linear correlation (r = 0.372; P < 0.05) between the xanthone content of the XEFs and their achieved α-glucosidase inhibition (ESI; Fig. S1†). XEF_1, with the highest mean xanthone content (480.8 g kg^{-1}), achieved the strongest mean α-glucosidase inhibition (72.2%), however the XEF with the lowest mean xanthone content (XEF_7; 222.57 g kg^{-1}) achieved a level of inhibition (mean = 67.5%) not far below that of XEF_1. There was a stronger linear correlation (r = 0.650; P < 0.001) between the benzophenone content of the BEFs and their achieved α-glucosidase inhibition. The BEF with the highest benzophenone content (BEF_3; 251.21 g kg^{-1}), achieved the strongest inhibition (36.1%), but—similar to the XEFs—the BEF with the lowest mean benzophenone content (BEF_5; 113.84 g kg^{-1}) was not the least effective.

The generally weak correlation between the phenolic content of the enriched fractions and their inhibitory activity against α-glucosidase indicates the likely contribution of other

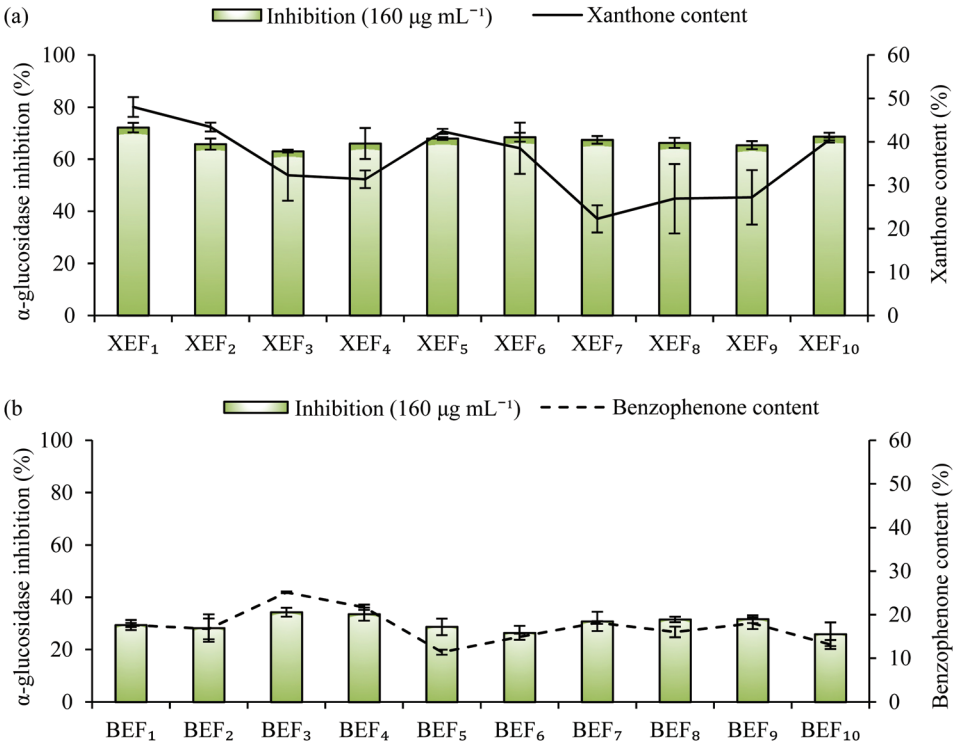

Fig. 3 Bar graphs indicating percentage inhibition of mammalian α-glucosidase achieved *in vitro* at 160 μg mL^{-1} by (a) ten xanthone-enriched fractions (XEFs) and (b) ten benzophenone-enriched fractions (BEFs), prepared from different batches of *Cyclopia genistoides* (n = 10). Fractions were prepared in triplicate from each batch (n = 10) and enzyme inhibition testing was performed in triplicate tested in triplicate. Data are presented as mean ± standard deviation (n = 3). Xanthone and benzophenone content of fractions are presented by the line graph.

factors to the observed inhibitory activity. This may include matrix effects due to the presence of other unidentified AGIs in the fractions or "impurities" that may interact with the enzyme and enhance or decrease the bioactivity. Tan *et al.*[36] used MARC to remove sugars and organic acids from crude extracts of black legumes, and the resultant fractions of purified and semi-purified polyphenols were more potent inhibitors of α-glucosidase than the original crude extracts. This could have been a consequence of concentrating the active AGIs in the extract, or of the removal of antagonists or non-inhibitors from the sample matrix. Higher or lower than expected inhibitory activity could also be the result of synergistic interaction between different AGIs in the same sample.

In vitro synergistic effects of combined α-glucosidase inhibitors

Testing of different combinations, *i.e.* fraction-fraction, fraction-compound and fraction or compound with acarbose allowed insight into potential synergy and the effect of composition on potency. For all combinations of acarbose with enriched fractions (XEF_0, BEF_0) or single phenolic compounds (combinations 1–6), the calculated CI fell within the range 0.36–0.63 at the 50 and 75% effect levels, which indicates synergistic interactions (Table 2).

Combinations of acarbose with mangiferin and isomangiferin showed the strongest synergistic effects (CI < 1) in general. Interestingly, the acarbose : XEF_0 combination displayed antagonism (CI = 1.24) at the 25% effect level despite the individual xanthones (mangiferin, isomangiferin) acting

Table 2 Chou–Talalay combination indices at the 25, 50 and 75% effect levels for 13 different combinations of mammalian α-glucosidase inhibitors

No.	Combination[a]	Concentrations[b] (μg mL^{-1})	Combination index (CI) at effect level		
			25%	50%	75%
1	Aca[c] : BEF_0[d]	2.5, 5, 10, 20, 40, 80, 160	0.925	0.603	0.492
2	Aca : XEF_0[e]	12.5, 25, 50, 100, 200, 400	1.240	0.630	0.478
3	Aca : MGF[f]	2.5, 5, 10, 20, 40, 80, 120	0.503	0.366	0.387
4	Aca : IMGF[g]	2.5, 5, 10, 20, 40, 80, 120	0.752	0.487	0.408
5	Aca : I3G[h]	5, 10, 20, 40, 80, 120	1.177	0.563	0.437
6	Aca : IDG[i]	5, 10, 20, 40, 80, 120	1.232	0.590	0.435
7	BEF_0 : XEF_0	12.5, 25, 50, 100, 200, 400	0.863	0.707	0.581
8	MGF : IMGF	10, 20, 40, 80	1.109	1.016	0.940
9	MGF : I3G	10, 20, 40, 80	0.775	0.904	1.060
10	IMGF : I3G	10, 20, 40, 80	1.096	1.009	0.950
11	IDG : I3G	10, 20, 40, 80	0.687	0.980	1.397
12	IDG : MGF	10, 20, 40, 80	0.902	0.902	0.905
13	IDG : IMGF	10, 20, 40, 80	0.948	1.017	1.109

[a] 1 : 1 combinations (μg mL^{-1}) of α-glucosidase inhibitors. [b] Concentrations of both inhibitors in the reaction volume for the indicated combination. [c] Acarbose. [d] Reference benzophenone-enriched fraction. [e] Reference xanthone-enriched fraction. [f] Mangiferin. [g] Isomangiferin. [h] 3-β-D-Glucopyranosyliriflophenone. [i] 3-β-D-Glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone.

synergistically with acarbose at the same effect level. This is a clear indication of matrix effects and that the activity of pure compounds, even as the major constituents of an extract, do not necessarily translate into the same activity when the extract is used. The reason for the switch in type of activity at the higher effect levels (50 and 75%) is not clear. Other methods should be applied to confirm results, *e.g.* Caesar and Cech[37] also proposed the use of a modified CI range to indicate the effects, *e.g.* CI > 4 would indicate antagonism instead of CI > 1.1. When acarbose was combined with I3G and IDG (combinations 5 and 6, respectively), the synergistic effects were generally less pronounced than for the xanthone-acarbose combinations. The synergism between acarbose and the benzophenones and xanthones explains in part the observed synergism between acarbose and the enriched *C. genistoides* fractions (combinations 1 and 2; CI < 0.630 at 50 and 75% effect levels).

Cyclopia genistoides extracts or enriched fractions will typically contain more than one identified compound with confirmed α-glucosidase inhibitory activity, not to mention some as yet unknown compounds that may potentially contribute to the overall effect, including synergistic, additive or antagonistic interactions with other AGIs or even augmentation by "inactive" compounds. Combination of phenolic compounds demonstrated mostly additive effects (combinations 8–13; Table 2), indicating that manipulation of the composition of the more potent XEF in terms of benzophenone content, has little value. In general, near-additive combined effects were observed (0.9 < CI < 1.1). CI values were above 0.9, with the exception of combination 9 (mangiferin : I3G) and 11 (IDG : I3G) at the 25% effect level. Interestingly, combining the benzophenones, I3G and IDG, resulted in possible antagonism at the 75% effect level (CI = 1.394) and synergism at the 25% effect level (CI = 0.687). Using the more conservative approach recommended by Caesar and Cech,[37] a CI range of 1.0–4.0 indicates an "indifferent" effect, with antagonistic effect higher than 4.0.

***In vitro* dose reduction of acarbose.** A major benefit of synergistic interaction between bioactive compounds is the potential to reduce the dose of one or more of the compounds while maintaining the same effect level, which may reduce the risk of toxicity, side effects and the development of drug resistance over time.[38–40] Acarbose is the most widely prescribed commercial AGI despite reports of gastrointestinal side effects (bloating, flatulence and diarrhoea) preventing its more widespread utilisation.[7] Reports of side effects vary widely amongst different individuals and population groups, since individual dietary compositions and gastrointestinal environments will affect how well commercial AGIs are tolerated. A typical acarbose treatment regime is normally prescribed at 3 × 50 mg per day,[41] with a maximum of 3 × 100 mg per day, as 200 mg is associated with a higher incidence of adverse effects of malabsorption.[42] There is a growing interest in finding less potent, natural alternatives to commercial AGIs, particularly if synergistic interaction with acarbose would allow for dose reduction benefits in addition to other potential health benefits associated with its phytochemical content.[43]

Another quantitative measure of synergism provided by the Chou–Talalay method is DRI, which is derived from CI, and represents the theoretical *x*-fold reduction, at a given effect level, in the dose of a particular agent in a synergistic combination. In general, DRI > 1 denotes synergism and DRI < 1 denotes antagonism, but in some cases, DRI may be greater than 1 despite the presence of antagonism according to the CI value (CI < 0.9). In such instances, the CI value should take precedence in classifying the type of interaction.[30] Table 3 lists the DRI for acarbose at the 25, 50 and 75% effect levels for all combinations containing acarbose (combinations 1–6). The greatest potential dose reductions (>six-fold) across all effect levels were achieved by combinations of acarbose with mangiferin or isomangiferin. A nearly 20-fold acarbose dose reduction was calculated for combinations 3 and 4 at the 75% effect level. Of the two enriched fractions of *C. genistoides*, BEF_0 showed less dose reduction potential in combination with acarbose (DRI = 1.15–3.57) compared with XEF_0 (DRI = 1.05–10.74). The acarbose dose reduction potential of BEF_0 and XEF_0 was more or less the same at the lower effect levels, but XEF demonstrated much greater dose reduction potential at the 75% effect level. CompuSyn-generated effect level *vs.* log (DRI) plots and isobolograms for combinations 1–13 are included as ESI (Fig. S2–14†).

Effect of batch-to-batch variation of raw plant material on acarbose dose reduction by fractions. In an industrial setting, one would expect large inherent variation in the xanthone and benzophenone content of different batches of *C. genistoides*, as plant breeding has not yet progressed to a cultivar. Furthermore, the effects of environmental stress on the phenolic composition have not yet been investigated extensively.[44] The effect of this natural variation on synergistic α-glucosidase inhibition with acarbose was investigated by testing combinations of acarbose with XEFs and BEFs prepared from 10 batches of plant material, harvested at two locations (Table 4).

Table 3 Acarbose dose reduction indices (DRIs) at the 25, 50 and 75% effect levels for combinations of acarbose with the xanthone- and benzophenone-enriched fractions of *Cyclopia genistoides* and four major phenolic compounds occurring in *C. genistoides* extract

		Acarbose dose reduction index (DRI) at effect level		
No.	Combination	25%	50%	75%
1	Aca[a] : BEF_0[b]	1.15	2.03	3.57
2	Aca : XEF_0[c]	1.05	3.36	10.74
3	Aca : MGF[d]	2.94	7.56	19.66
4	Aca : IMGF[e]	2.31	6.69	19.34
5	Aca : I3G[f]	1.00	3.16	9.94
6	Aca : IDG[g]	0.90	2.46	6.75

[a] Acarbose. [b] Reference benzophenone-enriched fraction. [c] Reference xanthone-enriched fraction. [d] Mangiferin. [e] Isomangiferin. [f] 3-β-D-Glucopyranosyliriflophenone. [g] 3-β-D-Glucopyranosyl-4-*O*-β-D-glucopyranosyliriflophenone.

Table 4 Acarbose dose reduction indices at the 25, 50 and 75% effect levels for combinations of acarbose with xanthone-enriched fractions (XEFs) and benzophenone-enriched fractions (BEFs) of *Cyclopia genistoides* produced (in triplicate) from ten batches of plant material

Fraction combined with acarbose[a]	Content in fraction[b] (g kg^{-1})		Acarbose DRI[c]		
	Benzophenones	Xanthones	25%	50%	75%
XEF_1[d]	35.64 ± 3.7	480.80 ± 23.0	2.25 ± 0.1	5.16 ± 0.1	11.87 ± 0.7
XEF_2	31.08 ± 2.7	434.35 ± 10.3	1.96 ± 0.1	4.18 ± 0.1	8.40 ± 0.7
XEF_3	78.30 ± 8.4	323.08 ± 58.8	2.15 ± 0.1	4.07 ± 0.2	7.73 ± 1.2
XEF_4	26.56 ± 6.0	320.64 ± 16.2	2.17 ± 0.1	4.44 ± 0.1	9.09 ± 0.7
XEF_5	15.91 ± 0.9	424.03 ± 5.9	2.15 ± 0.1	4.62 ± 0.1	9.96 ± 0.7
XEF_6	22.62 ± 0.6	385.34 ± 59.3	2.22 ± 0.2	4.69 ± 0.4	10.05 ± 2.2
XEF_7	22.72 ± 2.2	222.57 ± 31.1	2.22 ± 0.2	4.54 ± 0.1	9.30 ± 0.3
XEF_8	30.87 ± 4.0	269.14 ± 79.9	2.14 ± 0.2	4.41 ± 0.1	9.10 ± 0.9
XEF_9	33.87 ± 2.4	271.84 ± 62.8	2.08 ± 0.1	4.41 ± 0.1	9.40 ± 0.7
XEF_{10}	32.95 ± 1.6	404.31 ± 5.8	2.16 ± 0.2	4.61 ± 0.1	9.91 ± 0.8
BEF_1[e]	176.23 ± 4.7	Traces	2.53 ± 0.1	2.20 ± 0.1	1.92 ± 0.2
BEF_2	167.47 ± 23.9	Traces	2.08 ± 0.9	1.82 ± 0.3	1.98 ± 1.2
BEF_3	251.21 ± 1.91	nd	2.16 ± 0.1	2.56 ± 0.1	3.04 ± 0.4
BEF_4	217.05 ± 5.9	nd	1.72 ± 0.3	1.67 ± 0.2	1.64 ± 0.6
BEF_5	113.84 ± 5.8	Traces	1.79 ± 0.3	1.52 ± 0.2	1.35 ± 0.6
BEF_6	149.06 ± 7.0	Traces	2.07 ± 0.3	1.59 ± 0.0	1.25 ± 0.2
BEF_7	181.89 ± 3.2	Traces	2.04 ± 0.3	1.95 ± 0.1	1.92 ± 0.5
BEF_8	160.32 ± 12.3	Traces	2.57 ± 0.5	2.33 ± 0.2	2.22 ± 0.8
BEF_9	180.45 ± 13.5	Traces	1.79 ± 0.2	1.91 ± 0.3	2.10 ± 0.7
BEF_{10}	131.38 ± 10.3	nd	1.87 ± 0.3	1.59 ± 0.1	1.39 ± 0.3

[a] 1 : 1 combination in terms of μg mL^{-1} concentration in the reaction volume. [b] Enriched fractions previously analysed by HPLC-DAD (Miller *et al.*, 2020[16]). [c] Dose reduction index. [d] Xanthone-enriched fraction. [e] Benzophenone-enriched fraction. Fractions were prepared in triplicate from each batch (n = 10) and enzyme inhibition testing was performed in triplicate. Data are presented as mean ± standard deviation.

The acarbose DRI values indicate that the XEFs generally showed greater potential for acarbose dose reduction than the BEFs, confirming the trend observed for XEF_0 and BEF_0. XEF_1, with the highest xanthone content (480.80 g kg^{-1}), also had the highest mean acarbose DRI at all effect levels tested. Similarly, the BEF with the highest mean benzophenone content (BEF_3; 251.21 g kg^{-1}) had the highest DRI amongst the BEFs at all tested levels. These results suggest that an enriched fraction of *C. genistoides*, containing >190 g kg^{-1} xanthones, could potentially be used to achieve at least a theoretical four-fold acarbose dose reduction at effect levels >50%. The evidence of *in vitro* anti-diabetic effects and acarbose dose reduction activity such as presented here suggest that *in vivo* testing of acarbose-XEF fractions for their blood glucose lowering effects in an animal model would be of great interest. In a previous study,[45] the combination of acarbose with baicalein, a flavonoid found in *O. indicum*, showed synergistic inhibition against mammalian α-glucosidase *in vitro* (CI < 0.41). Subsequent *in vivo* experiments demonstrated that a combination of 1 mg kg^{-1} acarbose with 80 mg kg^{-1} baicalein synergistically reduced blood glucose levels in Kunming mice, with a hypoglycaemic effect roughly equivalent to 8 mg kg^{-1} acarbose, *i.e.* an eight-fold acarbose dose reduction.

Acarbose is a competitive inhibitor of α-glucosidase,[32] while mangiferin is a non-competitive inhibitor as shown for yeast α-glucosidase.[46] The latter study also demonstrated that mangiferin suppressed postprandial hyperglycemia in diabetic mice in the oral starch test, confirming its inhibitory effect on mammalian α-glucosidase. The severity of gastrointestinal side effects of pharmaceutical AGIs is typically dose-dependent, but the subjective nature of their side effects, and their close association with the complex gastrointestinal environment and dietary composition of the individual in question, make these side effects impossible to evaluate in animal models, and challenging in human studies.[41] A reliable evaluation of the effects of specific AGIs, or AGI combinations, on gastrointestinal side effects could only be accomplished in a tightly controlled clinical study, over a sufficiently long time period, with completely standardised diets. Even then, variation in the physicochemical gastrointestinal environments of different individuals could predispose some to severe side effects more so than others. In the absence of such comprehensive data, preclinical studies could provide more insight.

An unintentional, yet relevant effect of increased transit of partially digested carbohydrates into the colon, where bacterial fermentation causes symptoms of indigestion, is the introduction of other factors that may have a significant impact on the observed *in vivo* effects. Unabsorbed dietary polyphenols that reach the colon could exert a prebiotic effect by suppressing the growth of pathogenic bacteria and stimulating the growth of beneficial species.[47] It was found that short-chain fatty acids, including phenolic acids (microbial degradation products of polyphenols such as mangiferin)[48] that sustain the microbiome,[49] may also play a beneficial role in energy homeostasis in type 2 diabetes.[50]

Conclusions

This study has demonstrated for the first time the synergistic *in vitro* inhibition of mammalian intestinal α-glucosidase by combinations of acarbose with (1) the major bioactive compounds found in *C. genistoides*, and (2) enriched phenolic fractions of *C. genistoides*. The degree of synergism, indicated by the combination index of Chou–Talalay, differed depending on the effect level. These results highlight the potential of *C. genistoides* extract fractions, enriched in xanthones and benzophenones, for reducing the effective dose of acarbose required to prevent postprandial hyperglycaemia. This could prevent or alleviate the dose-related side effects of acarbose, which has been reported as a significant factor resulting in the discontinuation of treatment.

Conflicts of interest

There are no conflicts of interest to declare.

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