Heliyon 8 (2022) e10131

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Evaluation of anthoxanthins and their actions on digestive enzyme inhibition when used independently and in combination

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ARTICLE INFO

Keywords:
Anthoxanthins
Synergistic inhibition
Acarbose
α-amylase
α-glucosidase

ABSTRACT

Carbohydrate digestibility is a key determinant for elevated postprandial hyperglycemia (PPHG). Apart from dietary restrictions, one of the strategies to reduce PPHG is to limit the activity of carbohydrate digestive enzymes within the gastrointestinal tract in order to reduce monosaccharide absorption rates. The present work aimed to assess the inhibitory capabilities of digestive enzymes (e.g., α-glucosidase and α-amylase) by anthoxanthins when used independently, in combination with acarbose, or with a different anthoxanthin. Our results showed that quercetin, myricetin, and luteolin presented lower IC_{50} values than acarbose and inhibited α-glucosidase through mixed-type inhibition. On the other hand, acarbose when compared with these anthoxanthins, remained the most potent inhibitor of α-amylase. Combinatorial treatment (i) acarbose-quercetin and (ii) myricetin-luteolin showed synergistic activity (CI value less than 0.9) in α-glucosidase inhibition. An additive effect (CI value between 0.9 and 1.1) in α-glucosidase inhibition was observed when acarbose-myricetin, acarbose-luteolin or when a combination of two different anthoxanthins (quercetin-myricetin and quercetin-luteolin) was used. This study suggests the potential use of anthoxanthins as functional food ingredients to mitigate PPHG towards the management of T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common form of DM, and its growing prevalence appears to be associated with changing dietary habits and lifestyles (Burton-Freeman et al., 2019). Characterised by the impaired ability of insulin to lower blood glucose levels (Czech, 2017), T2DM is primarily a disorder of postprandial glucose regulation (Ceriello and Genovese, 2016). If left unchecked, postprandial hyperglycaemia (PPHG) increases the risk of both micro- and macrovascular complications (Sudhir and Mohan, 2002). Hence it is essential to manage PPHG and maintain blood glucose levels as close to normal levels as possible for the management of T2DM.

To suppress PPHG, one effective strategy is to slow down the absorption of glucose through the inhibition of principal digestive enzymes such as α-amylase and α-glucosidase which hydrolyse carbohydrates into monosaccharides (Teng and Chen, 2017). α-Amylase produced by the salivary glands and the pancreas, is the key enzyme that catalyses the hydrolysis of α-1,4-glucan linkages in starch and other related carbohydrates. Whereas α-glucosidase (mainly present in the brush border of human intestinal mucosal cells) is the most important enzyme for carbohydrate digestion that catalyses glucose from the nonreducing end of poly- or disaccharides by hydrolysing the α-1,4-glycosidic bond. Dietary starch and other related carbohydrates are digested by α-amylase to maltose, which is then further digested by α-glucosidase to glucose to be absorbed across the intestinal barrier aided by glucose transporters (Vocadlo and Davies, 2008). Hence, inhibition of α-glucosidase and α-amylase as pre-absorption determinants of carbohydrate metabolism, offers a complementary strategy to dietary control and other pharmacotherapy for the treatment of diabetic patients (Gong et al., 2020).

Anthoxanthins (flavones and flavonols) are a subgroup of flavonoids, similar to that of anthocyanins, that are present in fruits and vegetables (Han et al., 2007) and share the C6–C3–C6 flavan or 2-phenyl-benzodihydropyrane skeleton albeit in a less oxidized state (Ockermann et al., 2021). To date, anthoxanthins have been reported to exhibit digestive enzyme inhibiting capacity and, therefore, have the potential to attenuate PPHG and modulate T2DM (Kulkarni and Kamble, 2021; Naik and

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https://doi.org/10.1016/j.heliyon.2022.e10131
Received 24 April 2022; Received in revised form 22 June 2022; Accepted 27 July 2022

Kokil, 2013). The potential use of anthoxanthins as functional ingredients in food products can improve food quality through the attenuation of postprandial increases in blood glucose and blood insulin. Moreover, their combination with a commonly prescribed α-glucosidase inhibitor drug like acarbose for the management of patients with T2DM, may potentially minimize the undesirable side effects of the synthetic oral α-glucosidase inhibitor drug (Chiasson et al., 2002) by virtue of reducing its therapeutic dosage.

The present study evaluated the inhibitory activity of α-glucosidase and α-amylase by anthoxanthins and rationalized their modes of inhibition. We further examine their efficacy when used alone, in combination with acarbose, or in combination with a different anthoxanthin.

2. Materials and methods

2.1. Chemicals

The anthoxanthins namely myricetin, quercetin, luteolin, baicalein, chrysin, apigenin, and kaempferol were purchased from Runyu Biotechnology Co. Ltd. The identity and purity of the anthoxanthins were confirmed by nuclear magnetic resonance (NMR). 4-nitrophenol α-D-glucopyranoside (pNPG, N0493) and acarbose (A2485) were purchased from Tokyo Chemical Industry Co. Ltd. α-Glucosidase from *Saccharomyces cerevisiae* (G5003), α-amylase from human pancreas (A9972), 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG, 93834) were purchased from Sigma-Aldrich. All anthoxanthins were dissolved in dimethyl sulfoxide (DMSO, Thermo Fisher Scientific), with a final DMSO concentration of 2.5% in the reaction mixture for the enzymatic assays.

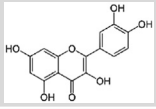
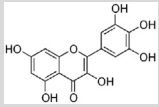
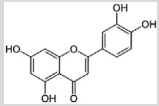
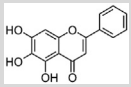
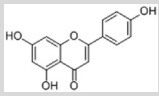
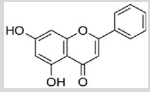
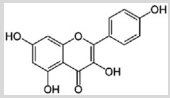
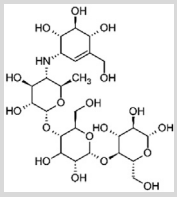
2.2. In vitro inhibition assay for α-glucosidase activity

α-glucosidase activity was measured by monitoring spectrophotometrically, at 405 nm, the conversion of pNPG into α-D-glucose and p-nitrophenol (Proenca et al., 2017). The enzyme and pNPG were dissolved in a 0.1 M phosphate buffer (pH 6.8) to give a final concentration of 0.05 U/mL and 600 μM, respectively, in the reaction mixture. In a 96-well plate, the enzyme was first pre-incubated at 37 °C for 5 min together with the tested compound. pNPG was subsequently added and the reaction mixture was then incubated at 37 °C for 30 min. To determine the IC_{50} value of each compound, the absorbance readings at the end of incubation was monitored and % activity was calculated using the formula $\frac{A}{B} \times 100\%$, where A is the absorbance in the presence of anthoxanthin, and B is the absorbance without anthoxanthin. Acarbose (0–2000 μM) was used as a positive control.

2.3. In vitro inhibition assay for α-amylase activity

α-amylase activity was measured by monitoring spectrophotometrically, at 405 nm, the conversion of CNPG into 2-chloro-4-nitrophenol and maltotriose (Proenca et al., 2019). The enzyme and CNPG were dissolved in a 0.04 M phosphate buffer with 0.007 M sodium chloride (pH 6.9) to give a final concentration of 0.3125 U/mL and 500 μM, respectively, in the reaction mixture. In a 96-well plate, the enzyme was first pre-incubated at 37 °C for 5 min together with the anthoxanthins. CNPG was subsequently added, and the reaction mixture was then incubated at 37 °C for 30 min. To determine the IC_{50} value of each compound, the absorbance readings at the end of incubation was monitored and %

Table 1. The subclass of anthoxanthins and their individual IC_{50} inhibition against α-glucosidase and α-amylase.

Anthoxanthins	Subclass	Chemical structure	IC_{50} against α-glucosidase (mean ± SD)	IC_{50} against α-amylase (mean ± SD)
Quercetin	Flavonol		10.92 μM ± 4.04 μM	28.78 μM ± 1.84 μM
Myricetin	Flavonol		17.78 μM ± 1.75 μM	51.60 μM ± 4.93 μM
Luteolin	Flavone		42.36 μM ± 7.72 μM	28.55 μM ± 1.41 μM
Baicalein	Flavone		303.37 μM ± 57.19 μM	1276.67 μM ± 1.06.54 μM
Apigenin	Flavone		Precipitated at 1000 μM (in vial)	Precipitated at 1000 μM (in vial)
Chrysin	Flavone		Precipitated at 400 μM (in vial)	Precipitated at 400 μM (in vial)
Kaempferol	Flavonol		Precipitated at 80 μM (in vial)	Precipitated at 80 μM (in vial)
Acarbose	Anti-diabetic drug		1037.6 μM ± 189.88 μM	0.83 μM ± 0.09 μM

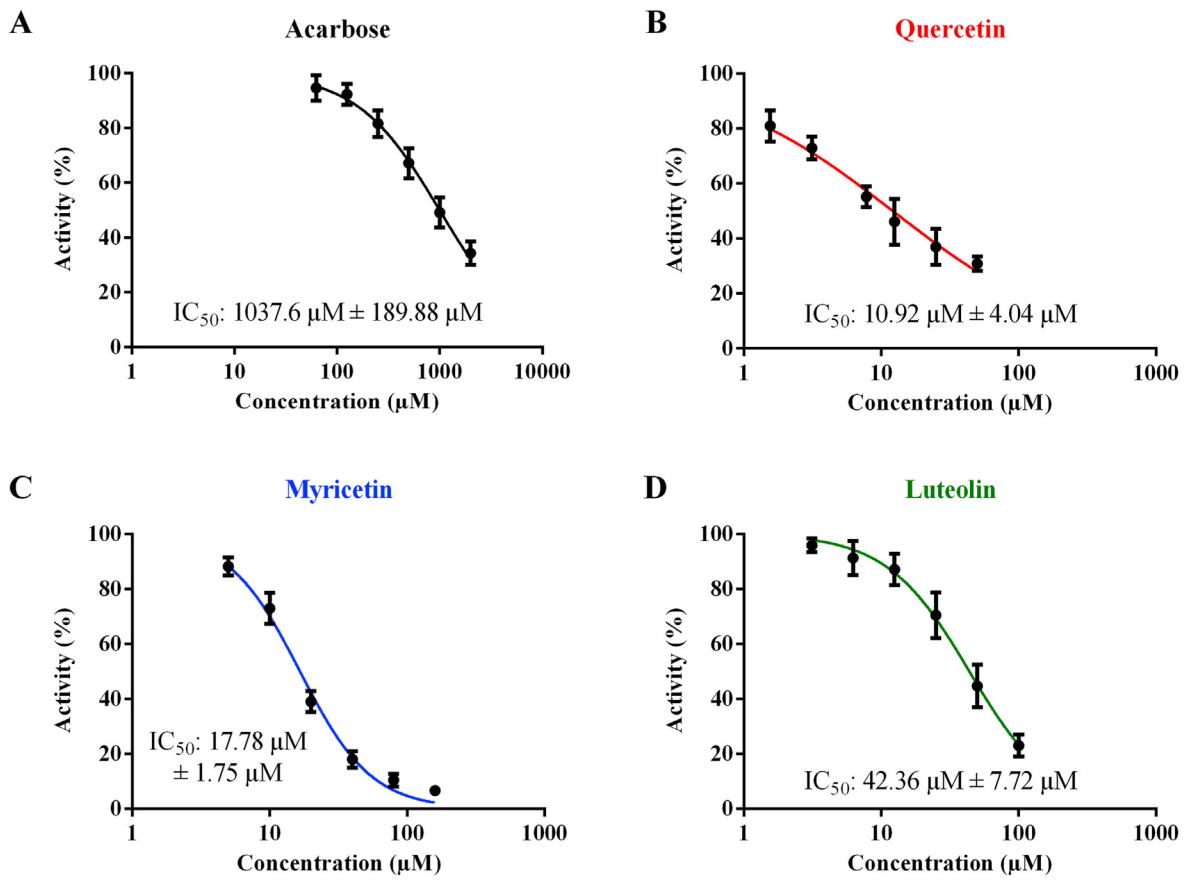


Figure 1. The inhibition of (A) acarbose, (B) quercetin, (C) myricetin or (D) luteolin against α-glucosidase. For the inhibition of α-glucosidase activity, the IC_{50} for acarbose was 1037.6 μM ± 189.88 μM. Quercetin, myricetin, and luteolin showed inhibitory activity, with IC_{50} = 10.92 μM ± 4.04 μM, 17.78 μM ± 1.75 μM, and 42.36 μM ± 7.72 μM respectively. The anthoxanthins (i.e., quercetin, myricetin, and luteolin) were more potent inhibitors of α-glucosidase than acarbose. The values were presented as means ± standard deviation.

activity was calculated using the formula $\frac{A}{B} \times$ 100%, where A is the absorbance in the presence of anthoxanthin, and B is the absorbance without anthoxanthin. Acarbose (0–2 μM) was used as a positive control.

2.4. Inhibitory kinetic analysis

The kinetic mode of inhibition was investigated on quercetin (0–25 μM), myricetin (0–40 μM), luteolin (0–100 μM) and acarbose (0–1000 μM). α-Glucosidase (0.05 U/mL) was dissolved in 0.1M phosphate buffer (pH 6.8) and incubated at 37 °C for 5 min together with these compounds of different concentrations. Subsequently, pNPG (300 μM, 600 μM, 900 μM, 1200 μM, and 1500 μM) were added and incubated at 37 °C for 15 min. The assay was measured by monitoring spectrophotometrically, at 405 nm, the conversion of pNPG into α-D-glucose and p-nitrophenol. The Michaelis-Menten constant (K_m) and the maximal rate of the enzyme (V_{max}) were determined by Lineweaver-Burk plot, which was established with double-reciprocal plot of the enzyme reaction velocity (v) against concentration of the substrate (pNPG). The data were then fitted into the different models of inhibition including those of competitive, non-competitive, uncompetitive, and mixed enzyme inhibition and analysed using GraphPad Prism Software 8.

2.5. Synergy evaluation in the α-glucosidase inhibition assay

The synergy evaluation of anthoxanthins-acarbose on α-glucosidase inhibition were grouped as (i) acarbose-quercetin (a mixture of 1000 μM acarbose and 25 μM quercetin), (ii) acarbose-myricetin (a mixture of 1000 μM acarbose and 40 μM myricetin), and (iii) acarbose-luteolin (a mixture of 1000 μM acarbose and 100 μM luteolin). A two-fold serial dilution of the acarbose-anthoxanthin samples were prepared and pre-incubated with α-glucosidase at 37 °C for 30 min, and the assay was measured by monitoring spectrophotometrically, at 405 nm, the conversion of pNPG into α-D-glucose and p-nitrophenol.

For the synergy evaluation of the combination of two different anthoxanthins, the combination was (i) quercetin-myricetin (a mixture of 25 μM quercetin and 40 μM myricetin), (ii) quercetin-luteolin (a mixture of 25 μM quercetin and 100 μM luteolin), and (iii) myricetin-luteolin (a mixture of 40 μM myricetin and 100 μM luteolin). A two-fold serial dilution of the mixed anthoxanthins samples were prepared and pre-incubated with α-glucosidase at 37 °C for 30 min and the assay was measured by monitoring spectrophotometrically, at 405 nm, the conversion of pNPG into α-D-glucose and p-nitrophenol. The combination index (CI) was calculated based on the median-effect principle developed by Chou and Talalay (Chou, 2006). The equation for calculating CI is as follows:

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2},$$

where $(D)_1$ and $(D)_2$ are concentrations of inhibitors that produce a certain level of inhibition in combination, and $(Dx)_1$ and $(Dx)_2$ are concentrations of inhibitors that produce the same level of inhibition when used alone. Based on the CI value, the interaction between the 2 inhibitors were then classified into synergistic (CI < 0.9), additive (CI = 0.9–1.1), or antagonistic (CI > 1.1).

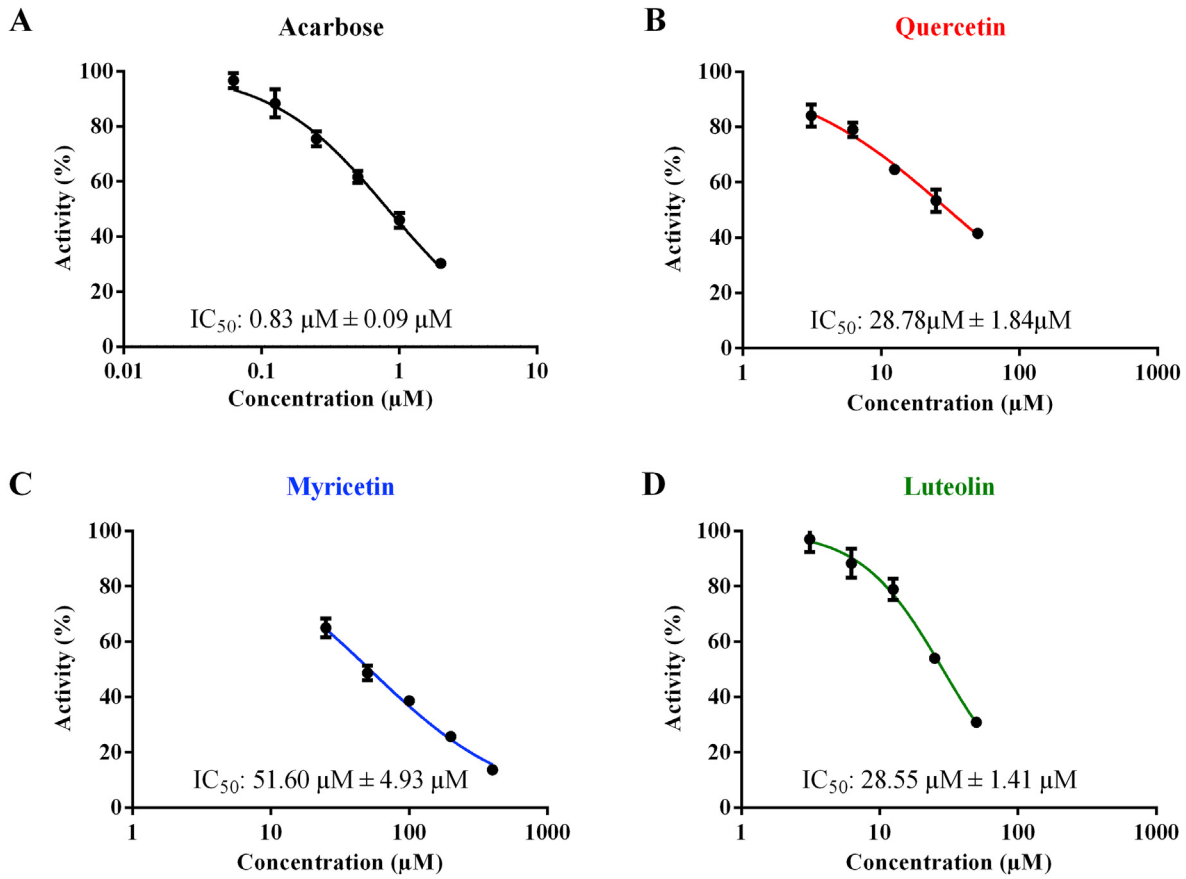


Figure 2. The inhibition of (A) acarbose, (B) quercetin, (C) myricetin or (D) luteolin against α-amylase. For the inhibition of α-amylase activity, the IC_{50} for acarbose was 0.83 μM ± 0.09 μM. Quercetin, myricetin, and luteolin showed inhibitory activity, with IC_{50} = 28.78 μM ± 1.84 μM, 51.60 μM ± 4.93 μM, and 28.55 μM ± 1.41 μM respectively. Acarbose was a more potent inhibitor of α-amylase than the anthoxanthins (i.e., quercetin, myricetin, and luteolin). The values were presented as means ± standard deviation.

3. Results and discussion

3.1. *In vitro inhibition of α-glucosidase and α-amylase activity by anthoxanthins*

Seven anthoxanthins were selected and their chemical structures were confirmed by NMR (data not shown). These anthoxanthins were tested on their inhibition on α-glucosidase and α-amylase activities. The IC_{50} values of each anthoxanthin and acarbose against the inhibition of α-glucosidase and α-amylase activity were presented in Table 1.

In our study, no inhibitory activity on α-glucosidase was observed with apigenin (at ≤1000 μM), chrysin (at ≤400 μM) and kaempferol (at ≤80 μM). These compounds dissolved in DMSO were observed to form precipitation upon mixing in pH 6.8 phosphate buffer at concentrations greater than 1000 μM, 400 μM, and 80 μM respectively (Kim et al., 2019; Sprachman and Wipf, 2012). Any activity at concentrations higher than their solubility limits would be inconclusive as such concentrations cannot be achieved without further modification. Therefore, only the flavonols (i.e., quercetin and myricetin), flavones (i.e., luteolin and baicalein) as well as acarbose were evaluated in subsequent enzyme inhibition experiments.

For the inhibition of α-glucosidase activity, the IC_{50} for acarbose was 1037.6 μM ± 189.88 μM. Quercetin, myricetin, luteolin and baicalein showed inhibitory activity, with IC_{50} = 10.92 μM ± 4.04 μM, 17.78 μM ± 1.75 μM, 42.36 μM ± 7.72 μM and 303.37 μM ± 57.19 μM respectively (Figure 1). Consistent with published studies (Li et al., 2018), some anthoxanthins were more potent inhibitors of α-glucosidase than acarbose, allowing them to exhibit pharmacological relevant effects, even if used at low concentrations found commonly in food.

For the inhibition of α-amylase activity, acarbose remains superior with IC_{50} at 0.83 μM ± 0.09 μM. Luteolin, quercetin, myricetin and baicalein showed inhibitory activity, with IC_{50} = 28.55 μM ± 1.41 μM, 28.78 μM ± 1.84 μM, 51.60 μM ± 4.93 μM and 1276.67 μM ± 106.54 μM respectively (Figure 2).

The inhibitory effect of quercetin, myricetin, and baicalein against α-glucosidase and α-amylase shows the same rank order (i.e., quercetin > myricetin > baicalein) with the exception for luteolin and acarbose. Acarbose was unique in demonstrating strong and selective inhibitory effect against α-amylase but much weaker against α-glucosidase. Also consistent with published literatures (Şöhretoğlu and Sari, 2020; Tadera et al., 2006), flavonols (i.e., quercetin and myricetin) compared with their flavone analogues were generally stronger inhibitors of α-glucosidase. Potentially, this is due to the presence of the extra hydroxyl group at position 3 of the C ring (Proenca et al., 2017; Şöhretoğlu and Sari, 2020) which may have enhanced their interaction with the enzyme.

Interestingly, luteolin is a flavone described by Kim et al. (2000) and Zhao et al. (2021) to effectively inhibit α-amylase despite being less potent than acarbose. This may be due to its two hydroxyl groups at C3′ and C4′ that give it an optimal conformation to form more hydrogen bonds at the active site of the enzyme, making luteolin possess a higher binding energy with α-amylase. The exact understanding of the inhibitory actions of luteolin on α-amylase and its potential uses remain to be further explored.

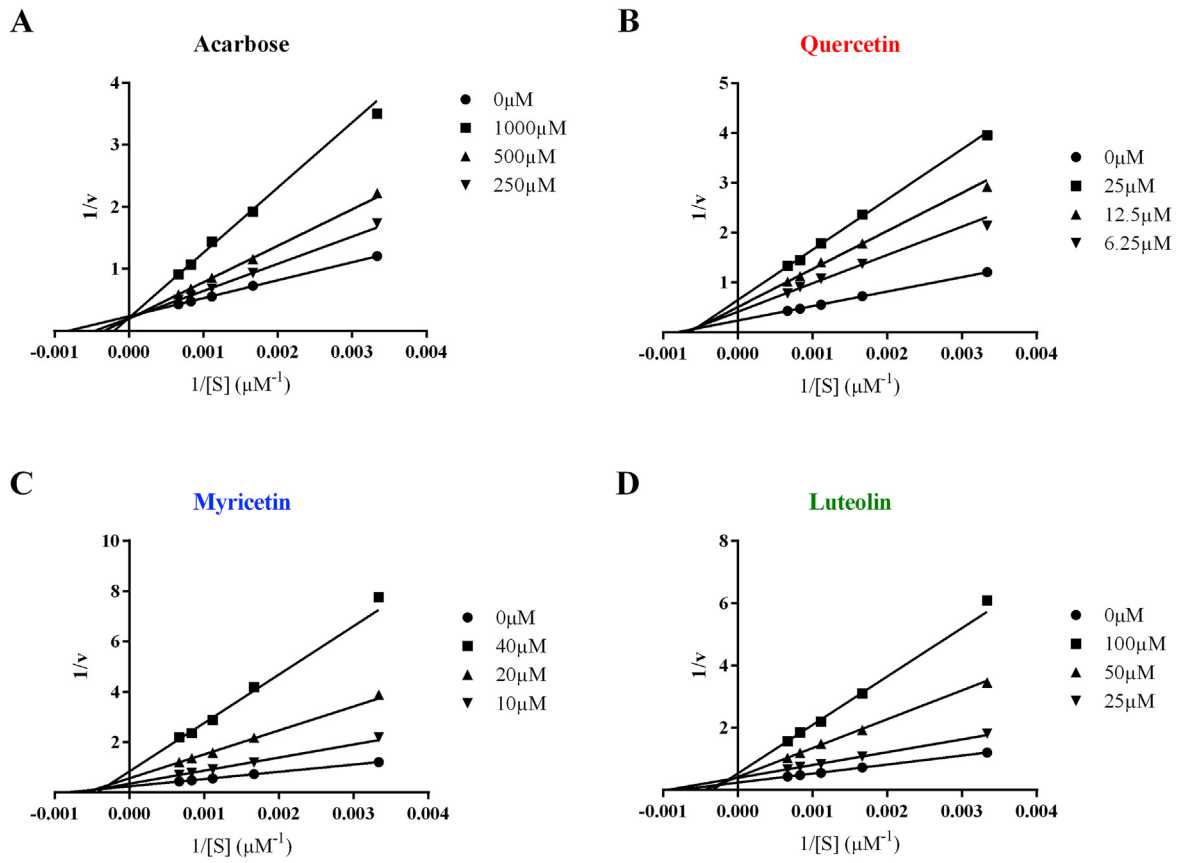


Figure 3. Lineweaver-Burk plots of α-glucosidase inhibition by (A) acarbose, (B) quercetin and (C) myricetin and (D) luteolin. Acarbose induced a competitive inhibition pattern against α-glucosidase with Ki = 483.6 μM ± 116.7 μM. While quercetin, myricetin and luteolin showed mixed inhibition patterns against α-glucosidase, with K_i = 7.71 μM ± 2.36 μM, 9.81 μM ± 2.29 μM and 30.44 μM ± 3.34 μM respectively. The Ki values were expressed as means ± standard deviation.

3.2. The type of inhibition by quercetin, myricetin, luteolin and acarbose on α-glucosidase

To characterize the mode of inhibition by quercetin, myricetin, luteolin, enzyme inhibition kinetics experiments were performed, and critical parameters were derived using the Lineweaver-Burk double reciprocal plots in Figure 3. In our findings, acarbose induced a competitive inhibition pattern against α-glucosidase with K_i = 483.6 μM ± 116.7 μM. The results were consistent with previous reports (Liu et al., 2019, 2020), where the K_m value of acarbose increased with increased acarbose concentration but there were no significant changes in the V_{max} value observed.

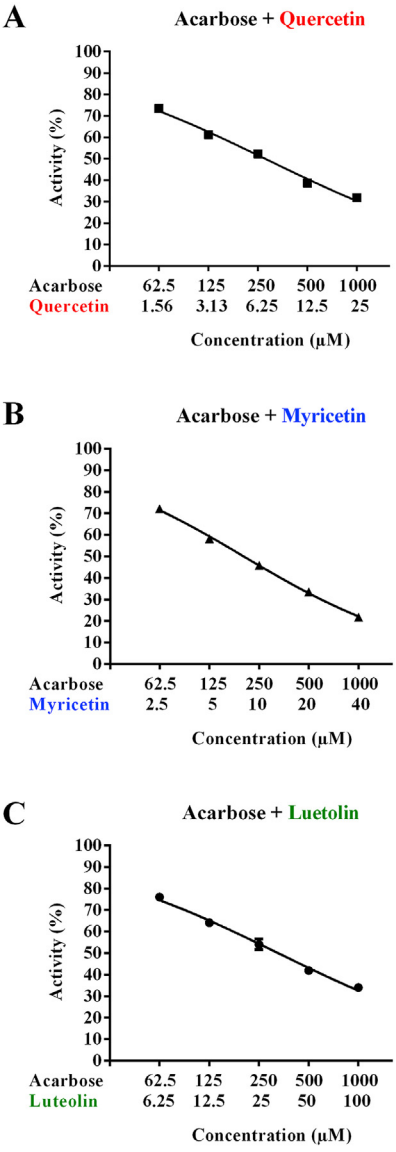
In contrast with acarbose, quercetin, myricetin and luteolin showed mixed inhibition patterns against α-glucosidase, where the intersection of the kinetic curve lies above the abscissa and to the left of the ordinate. The K_i of quercetin, myricetin and luteolin were calculated to be 7.71 μM ± 2.36 μM, 9.81 μM ± 2.29 μM and 30.44 μM ± 3.34 μM respectively. The smaller value of the constant suggested the stronger binding and more potent inhibition of quercetin and myricetin over luteolin on the α-glucosidase enzyme. This was consistent with our α-glucosidase inhibition assay experiment, which again demonstrated the importance of hydroxyl substitution of B ring and 3-OH of C ring of flavonoids on α-glucosidase inhibition activity (Proenca et al., 2017; Xu, 2010). Particularly, flavonoid compounds consisting the hydroxyl groups at the B ring were found to interact with the active-site residues of α-glucosidase, and the presence of 3-OH of C ring could contribute to maintaining the proper binding orientation of the enzyme, thus are important factors contributing to the inhibition activity of α-glucosidase (Kumar et al., 2010).

In addition, quercetin, myricetin and luteolin, which indicated a mixed-type of inhibition, suggested that these anthoxanthins could bind to free α-glucosidase as well as to α-glucosidase–substrate complexes, possibly interacting at or beyond the active site. Despite structural differences in the positions and presence of hydroxyl groups, the anthoxanthin scaffold consistently (at least for the 3 that have been tested) produces a mixed-mode inhibition. Such observation through molecular dynamics simulations were demonstrated by Liu et al. (2020) and Xu et al. (Xu, 2010), where structurally different compounds conferred differing inhibitory mechanisms. This includes the strength of the binding interaction between a single biomolecule which binds to the enzyme and the enzyme–substrate complexes, suggesting the existence of multiple enzyme–inhibitor binding sites or the involvement of multiple structural binding modes at the same site.

Clinically, acarbose is already used as an antidiabetic drug, based on its well-established mechanism of action as a competitive inhibitor for α-glucosidase (Liu et al., 2020). Yet this study revealed the stronger inhibitory potency of the anthoxanthins (quercetin, myricetin and luteolin), exploiting a different binding mode as mixed function inhibitors. This profile presents unique opportunities for synergism, as it is conceivable that orthogonal binding (distinct from the binding pocket for acarbose binding) could take place simultaneously to enhance the overall inhibitory and consequently, the putative therapeutic action.

3.3. Combined inhibition of α-glucosidase by quercetin, myricetin or luteolin with acarbose as potential pharmaceutical use

To investigate the potential therapeutic applications of anthoxanthins, the combined effects of anthoxanthins with acarbose and on inhibition of α-glucosidase were studied. In our findings, the combination of quercetin and acarbose showed synergistic inhibition against the α-glucosidase enzyme (Figure 4A), and an additive inhibition against the



IC_{50}	CI	Interactions
268.49 ± 2.24μM Acarbose + 6.71 ± 0.06μM Quercetin	0.88 ± 0.039	Synergistic inhibition

IC_{50}	CI	Interactions
230.34 ± 1.59μM Acarbose + 9.21 ± 0.06μM Myricetin	0.93 ± 0.031	Additive inhibition

IC_{50}	CI	Interactions
293.23 ± 2.05μM Acarbose + 29.32 ± 0.20μM Luteolin	0.96 ± 0.022	Additive inhibition

Figure 4. The inhibition of the combination of acarbose with (A) quercetin, (B) myricetin or (C) luteolin against α-glucosidase. The combination required to produce 50% inhibition of the activity of α-glucosidase was (A) 268.49 μM ± 2.24 μM acarbose and 6.71 μM ± 0.06 μM quercetin, (B) 230.34 μM ± 1.59 μM acarbose and 9.21 μM ± 0.06 μM myricetin, and (C) 293.23 μM ± 2.05 μM acarbose and 29.32 μM ± 0.20 μM luteolin. Using Chou-Talalay CI method (Chou, 2006), the CI calculated for their combination at this level of inhibition was 0.88 ± 0.039, 0.93 ± 0.031 and 0.96 ± 0.022 respectively. The combination of quercetin with acarbose showed synergistic inhibition against the α-glucosidase enzyme. And additive inhibition against the α-glucosidase enzyme were observed with acarbose-myricetin and acarbose-luteolin. The values were presented as means ± standard deviation.

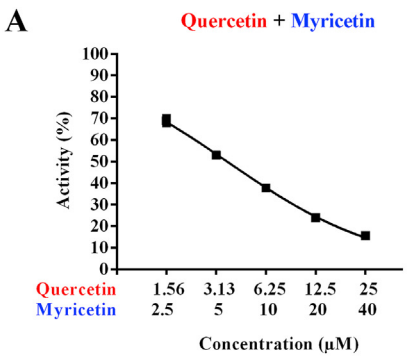
α-glucosidase enzyme was observed for acarbose-myricetin (Figure 4B) and acarbose-luteolin (Figure 4C). The combination of these anthoxanthins was found to strongly enhance the inhibition against α-glucosidase when compared to the compounds individually used alone. Acarbose-quercetin (268.49 μM ± 2.24 μM acarbose and 6.71 μM ± 0.06 μM quercetin) was required to produce 50% inhibition of the activity of α-glucosidase and the CI for their combination at this level of inhibition was 0.88 ± 0.039, suggesting synergistic inhibition. Additive inhibition of α-glucosidase was observed when myricetin and acarbose were used in combination. This combination gives a 50% inhibition of the activity of α-glucosidase at 230.34 μM ± 1.59 μM acarbose and 9.21 μM ± 0.06 μM myricetin, and the CI for their combination at this level of inhibition was 0.93 ± 0.031. Similarly, luteolin and acarbose in combination showed additive inhibition of α-glucosidase. The combination of luteolin and acarbose required to produce 50% inhibition of the activity of α-glucosidase was 293.23 μM ± 2.05 μM acarbose and 29.32 μM ± 0.20 μM luteolin, and the CI for their combination at this level of inhibition was 0.96 ± 0.022.

Concerning the interaction between anthoxanthins and acarbose, their inhibitory activity against α-glucosidase was more effective than when acarbose was used alone. A similar result was observed by several studies (Yang et al., 2021; Zhang et al., 2017a, 2017b) on the inhibition of α-glucosidase, where compounds that display mixed or non-competitive behaviour were combined with acarbose; a competitive α-glucosidase inhibitor, the co-inhibition effect was much higher than when acarbose was used individually, suggesting different binding sites of the two inhibitors, which prevented binding competition and potentiated overall inhibition (Zhang et al., 2017b).

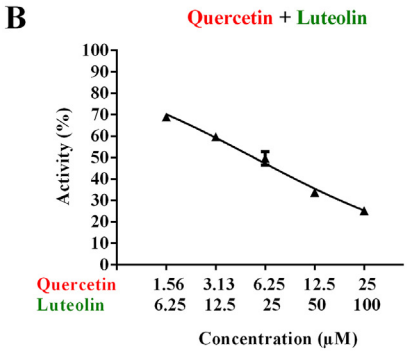
It is well established that the risk of diabetic complications is reduced through the control of PPHG (Faerch et al., 2018; Madsbad, 2016; Monnier et al., 2011). And the strategy for enhancing the inhibition of carbohydrate digestive enzymes (i.e., α-amylase and α-glucosidase) without adding pill burden, could be regarded as an efficient approach. Therefore, the incorporation of anthoxanthins with acarbose can be very beneficial as it could not only reduce the toxicity of acarbose but might possibly increase the overall therapeutic effect for the management of diabetes.

3.4. Combined inhibition of α-glucosidase by quercetin + myricetin, quercetin + luteolin or myricetin + luteolin as potential supplements and dietary use

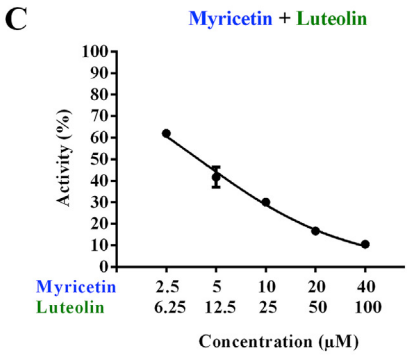
To investigate the potential applications of anthoxanthin combinations in supplements and dietary use, the combined effects of two different anthoxanthins on inhibition of α-glucosidase were studied. The



IC_{50}	CI	Interactions
4.51 ± 0.10μM Quercetin + 7.22 ± 0.16μM Myricetin	0.94 ± 0.013	Additive inhibition



IC_{50}	CI	Interactions
5.69 ± 0.13μM Quercetin + 22.76 ± 0.52μM Luteolin	0.96 ± 0.037	Additive inhibition



IC_{50}	CI	Interactions
6.41 ± 0.17μM Myricetin + 16.02 ± 0.43μM Luteolin	0.81 ± 0.004	Synergistic inhibition

Figure 5. The inhibition of the combination of (A) quercetin + myricetin, (B) quercetin + luteolin or (C) myricetin + luteolin against α-glucosidase. The combination required to produce 50% inhibition of the activity of α-glucosidase was (A) 4.51 μM ± 0.10 μM quercetin and 7.22 μM ± 0.16 μM myricetin, (B) 5.69 μM ± 0.13 μM quercetin and 22.76 μM ± 0.52 μM luteolin, and (C) 6.41 μM ± 0.17 μM myricetin and 16.02 μM ± 0.43 μM luteolin. Using Chou-Talalay CI method (Chou, 2006), the CI calculated for their combination at this level of inhibition was 0.94 ± 0.013, 0.96 ± 0.037 and 0.81 ± 0.004 respectively. The combination of luteolin with myricetin showed synergistic inhibition against the α-glucosidase enzyme. And additive inhibition against the α-glucosidase enzyme were observed with quercetin-myricetin and quercetin-luteolin. The values were presented as means ± standard deviation.

combination of myricetin (40 μM) and luteolin (100 μM) (Figure 5C), required to produce 50% inhibition of the activity of α-glucosidase was 6.41 μM ± 0.17 μM myricetin and 16.02 μM ± 0.43 μM luteolin, and the CI for their combination at this level of inhibition was 0.81 ± 0.004, suggesting synergistic inhibition. However, when myricetin (40 μM) was combined with luteolin (200 μM and 300 μM; data not shown), the CI for their combination were 0.93 and 0.98 respectively, showing an additive inhibition of α-glucosidase. Additive inhibition of α-glucosidase was observed when the flavone luteolin was used in combination with quercetin (Figure 5B) as well as when the two flavonols; quercetin and myricetin were combined (Figure 5A). Their combination gives a 50% inhibition of the activity of α-glucosidase at 5.69 μM ± 0.13 μM quercetin and 22.76 μM ± 0.52 μM luteolin, and at 4.51 μM ± 0.10 μM quercetin and 7.22 μM ± 0.16 μM myricetin with CI for their combination at this level of inhibition being 0.96 ± 0.037 and 0.94 ± 0.013 respectively.

In our findings, the flavone luteolin when used in combination with myricetin showed synergistic inhibition against the α-glucosidase enzyme (Figure 5). It appeared that synergism was stronger when a less potent anthoxanthin were combined. In particular, the flavone luteolin produced an IC_{50} of 42.36μM ± 7.72 μM when used alone on α-glucosidase inhibition compared to its combination with the flavonols, quercetin or myricetin, which produced an IC_{50} of 28.45 μM and 22.43 μM respectively. Similarly, this was also observed when the less potent flavonol myricetin (IC_{50} = 17.78 μM ± 1.75 μM) was used in combination with a more potent flavonol quercetin (IC_{50} = 10.92 μM ± 4.04 μM) which produced an IC_{50} of 11.73 μM ± 0.26 μM on α-glucosidase inhibition.

The pronounced synergism between anthoxanthins (i.e., myricetin-luteolin), which are superior to that of the combinations with acarbose, is a clinically significant one. It suggests the possibility that such compounds may be used as a pre-diabetic management PPHG, even before pharmaceutical agents like acarbose are applied for treatment (Vinholes and Vizzotto, 2017). Our results also lend weight to the hypothesis that mixed-type inhibition modes, may diversify and strengthen the inhibition on α-glucosidase activity to achieve greater pharmacological advantage. But to fully comprehend the binding mode for compound optimization, future *in silico* molecular docking simulation studies may help better understand the mode of synergistic inhibition of these compounds and their inhibitory mechanisms on α-glucosidase.

4. Conclusion

Our results demonstrated that quercetin, myricetin or luteolin, which showed to be a mixed-type inhibition of α-glucosidase, could be

promising α-glucosidase inhibitors for anti-diabetic approaches. The synergistic mode of actions observed with the combined of acarbose with quercetin as well as with the combined of myricetin and luteolin on α-glucosidase inhibition suggest their potential for pharmaceutical and dietary development for their use to synergistically prevent as well as to control T2DM. Potential applications for these results could be nutritional recommendations made to supply the daily diet with foods containing high amounts of the studied anthoxanthins, such as onions and citrus fruits (Horwitz, 2018). Alternatively, supplementation of the purified substances could be used as a nutraceutical approach in tandem with pharmacotherapy and even as a means to reduce the dosage of pharmacotherapeutic agents. The need for continual search for new α-glucosidase inhibitors, particularly from natural resources, is still necessary to provide more candidates of drug choices for potential future development of flavonoid-based drugs.

Declarations

Author contribution statement

Yong Qin Koh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yu Ang Desmond Sin, Hengyang Justin Rong, and Teng Hui Sean Chua: Performed the experiments; Analyzed and interpreted the data.

Si-Han Sherman Ho and Han Kiat Ho: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

Assoc Prof Han Kiat Ho was supported by National University of Singapore [R148-000-318-114 & R148-000-295-114].

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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