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Inhibition of the carbohydrate-hydrolyzing enzymes α-amylase and α-glucosidase by hydroxylated xanthones

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Xanthones are oxygen-containing heterocyclic compounds that exhibit a wide range of biological and pharmacological properties. Some natural and synthetic derivatives have been identified for their antidiabetic profile, mainly as α-glucosidase inhibitors. However, studies concerning the inhibition of both carbohydrate-hydrolyzing enzymes α-amylase and α-glucosidase are scarce. Thus, in order to identify some of these dual-target antidiabetic agents, a series of new synthetic xanthones were evaluated together with their commercial parents mangiferin (**4**), α-mangostin (**5**) and γ-mangostin (**6**). The results showed that xanthones exhibited a systematic stronger inhibition against α-glucosidase rather than for α-amylase. Derivatives **2c**, **3a** and **3b**, bearing one catechol moiety, were the most active inhibitors of α-amylase, while xanthones **2c**, **3b** and **3c** were the most active against α-glucosidase activity, with IC_{50} values lower than 10 μM. These findings suggest that the substitution pattern of the xanthone scaffold modulated the inhibitory activity of these compounds, and some structure–activity relationships could be established for both assays. In addition, the type of inhibition was also studied, and the results indicate a competitive type of inhibition for α-amylase activity by xanthones **2c**, **3b**, **3c** and γ-mangostin (**6**). On the other hand, non-competitive inhibition mechanisms can be ascribed for all xanthones **1–6** against α-glucosidase. The present work can open a promising area of research based on the design of novel xanthone derivatives, based on natural ones, for targeting key enzymes involved in glucose metabolism and therefore in the management of type 2 diabetes mellitus.

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1. Introduction

Diabetes mellitus (DM) is an endocrinological disorder characterized by hyperglycemia, resulting directly from insufficient pancreatic insulin secretion, coupled to impaired insulin response from target tissues such as muscle, liver and fat (a condition termed insulin resistance). According to the International Diabetes Federation (IDF), in 2019 there were 436 million (20–79 years old) diabetics worldwide, a number predicted to rise every year to an estimated number of 578 million in 2030 and 700 million in 2045. From the survey, more than 55% of diabetics have not been diagnosed and around 5 million people die of diabetes-related diseases every year.[1]

DM can be divided into four types: type 1, type 2, gestational and other specific types. Type 1 is the insulin-dependent condition more often arising in youth, after autoimmune destruction of the hormone secreting pancreatic islet β cells. It accounts for 5–10% of the total diabetics and is associated with children and adolescents who require daily insulin injection for maintaining their normal life. Type 2 DM (formerly known as non-insulin-dependent, or adult-onset) is known as the most common type of diabetes, comprising 90% of the total diabetics. It is associated either with insulin resistance or insulin secretion disorders, generally accompanied by societal onerous morbid complications such as diabetic nephropathy, retinopathy, chronic vascular disease, peripheral neuropathy, and in severe cases, death. Gestational diabetes can be developed at any stage of pregnancy and usually disappears after childbirth. It occurs when the body cannot produce enough insulin and represents a great risk of morbidity and mortality to the mother and the fetus.[2–5]

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Currently, treatment and/or management of DM include insulin (the pancreatic hormone responsible for regulating blood glucose levels) and non-insulin therapeutic options, in combination with a suitable diet and a balanced lifestyle. In the first case, there are short-, medium- and long-acting insulin injectable-form strategies. For the non-insulin therapy, several commercially available oral antidiabetic drugs have been developed over the years. It can include biguanides, sulfonamides, meglitinides, thiazolidinediones, α-glucosidase inhibitors, glucagon-like peptide-1 receptor agonists, protein tyrosine phosphatase 1B inhibitors, dipeptidyl peptidase IV inhibitors, and sodium-glucose cotransporter-2 inhibitors, among others.[6–9] In the present study we focused on therapeutic options that are involved in the first stages of carbohydrate digestion, namely through the inhibition of α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) hydrolytic enzymes, retarding the absorption of glucose and thereby suppressing postprandial hyperglycemia.[10]

Carbohydrate digestion begins in the mouth through the action of the salivary α-amylase, responsible for the process of breaking down α-(1,4)-glycosidic bonds of dietary carbohydrates such as starch to a series of branched glucans and small linear glucans. This mixture (chyme) enters the stomach and the action of the salivary α-amylase gets halted in the new acidic environment. The process is nonetheless resumed in the upper part of the small intestine by the release of the pancreatic enzyme α-amylase. Further catalytic combination with α-glucosidase enzyme results in di-, tri- and other oligosaccharides. Finally, free glucose is obtained through the action of the α-glucosidase located in the brush borders of the enterocytes of the jejunum, which can cleave α-(1,4)-glycoside bonds at the non-reductive end of oligosaccharides, and also α-(1,6)-glycoside bonds, albeit slowly. In this sense, targeting α-amylase and α-glucosidase enzymes would prevent the production of exceeding assimilable quantities of glucose and provide an efficacious approach to maintain normoglycemia in type 2 DM.

Acarbose, miglitol and voglibose are three α-glucosidase inhibitors clinically approved to manage blood-glucose levels.[9,11,12] Among these, acarbose, which also inhibits the action of α-amylase, is by far the most prescribed oral medication.[13] Despite the high inhibitory potential, serious side effects have been associated with this therapy that includes abdominal distension, flatulence and diarrhea, most of them due to the accumulation of undigested carbohydrates in the large intestine.[14] These adverse effects have been related to the higher inhibition of salivary and pancreatic α-amylase, when compared with α-glucosidase, which results in an excessive accumulation of undigested carbohydrates in the large intestine, culminating in the observed gastrointestinal side effects. Therefore, there is an urgent need to find newer and safer selective inhibitors of α-glucosidase able to modulate type 2 DM.[10,15–17]

Xanthones are oxygen-containing secondary metabolites known for their multiple pharmacological activities such as anti-inflammatory, antimicrobial, antioxidant and antitumor effects.[18–20] Recent studies further pointed to the inhibitory activity of several natural and synthetic xanthone derivatives.[21–23] Accordingly, phytochemical studies of some edible species, mainly belonging to the Mangifera and Garcinia genus, have identified xanthones as responsible for the inhibition of α-amylase and α-glucosidase enzymes. These findings stimulated the pursuit for novel bioactive compounds, particularly through the synthesis of prenylated and oxygenated analogues, which have revealed their effectiveness in suppressing the enzymatic activity, mainly against α-glucosidase.[21]

To the best of our knowledge, studies reporting the inhibitory potential together with the inhibition type of this class of compounds against both carbohydrate-hydrolyzing enzymes α-amylase and α-glucosidase are scarce or still inexistent.

In this sense, the inhibitory activity and the type of inhibition of a panel of nine new synthetic xanthones together with their commercial parents, mangiferin (**4**), α-mangostin (**5**) and γ-mangostin (**6**) (Fig. 1), against α-amylase and α-glucosidase activities are herein described.

2. Materials and methods

2.1. Materials and reagents

The following reagents were obtained from Sigma-Aldrich Co. LLC (St Louis, MO): α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae*, α-mangostin (**5**), γ-mangostin (**6**), acarbose, 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3), dimethylsulfoxide (DMSO), mangiferin (**4**), *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG), sodium hydrogen phosphate and sodium dihydrogen phosphate. Xanthones **1–3a–c** (Fig. 1) were synthesized as previously described in the literature.[24] For the enzymatic assays, xanthones **1–6** were dissolved in DMSO (the final concentration of DMSO in the reaction mixture was 4.76%). The amount of DMSO used had no interference with the assays. A multimode microplate reader (Synergy HT, BIO-TEK) with a temperature control capacity was used to record the spectrophotometric readings in all the assays.

2.2. *In vitro* α-amylase inhibitory activity assay

The α-amylase inhibition assay was performed according to reported methods with slight modifications.[25] α-Amylase mediates the hydrolysis of the substrate CNPG3 into 2-chloro-4-nitrophenol (CNP), 2-chloro-4-nitrophenyl-α-D-maltoside (CNPG2), maltotriose and glucose. The amount of CNP formed, which is proportional to the activity of α-amylase, was measured spectrophotometrically at the wavelength of 405 nm.

Briefly, in a 96-well plate, the enzyme (0.1 U mL^{-1}) dissolved in 20 mM phosphate buffer (pH 6.8) was incubated at 37 °C for 10 min with each one of the xanthones **1–6** (0–200 μM). Afterwards, the substrate CNPG3 (500 μM) was added, and the subsequent catalytic reaction was followed for another 30 min. Changes in the absorbance values between 5

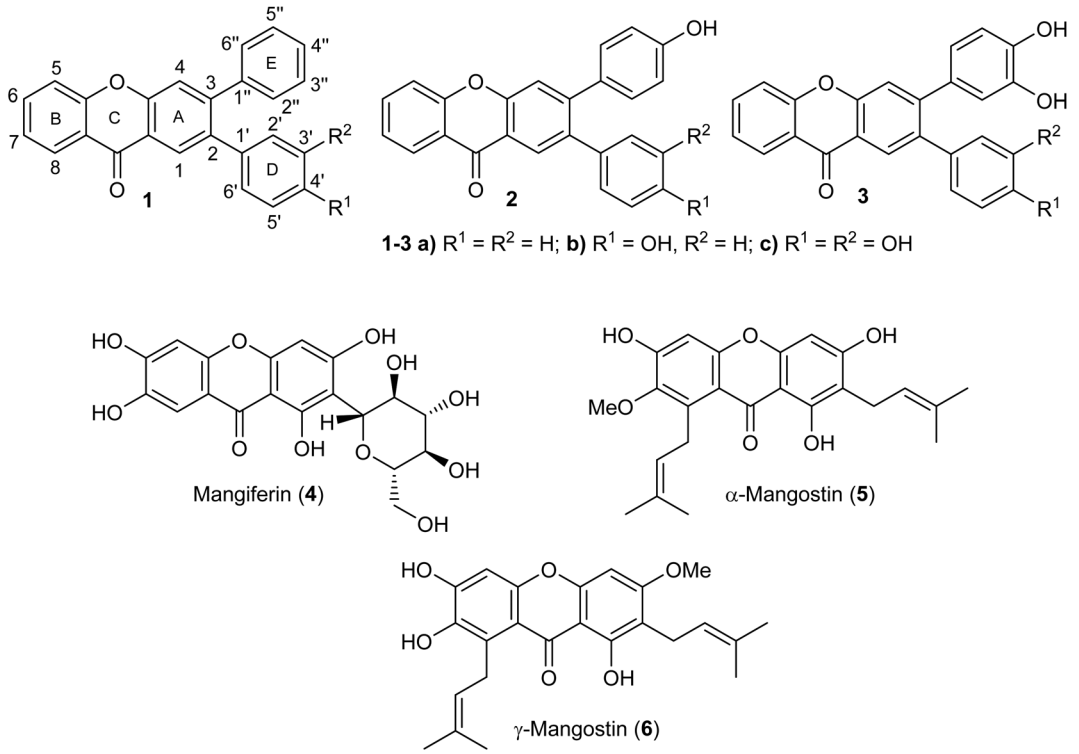


Fig. 1 Chemical structures of the tested xanthones **1–6**.

and 20 min enabled the calculation of kinetic values to the corresponding slopes. The obtained results, for which the standard error of mean (SEM) is also stated, are expressed as % inhibition of α-amylase activity (eqn (1)) and represent data averages of at least three independent experiments. Acarbose (0–5 μM) was used as the positive control.

$$\text{Inhibition}(\%) = \frac{\text{Slope}_{\text{sample}} - \text{Slope}_{\text{blank}}}{\text{Slope}_{\text{control}} - \text{Slope}_{\text{blank}}} \times 100 \qquad (1)$$

2.3. Inhibitory kinetic analysis of α-amylase

Inhibitory kinetic analyses were performed for all tested xanthones, except for **1b**, **4** and **5**, which showed residual activity in preliminary assays at the highest tested concentration (200 μM for **1b** and **4** and 90 μM for xanthone **5**). The tested concentrations were between 0.25–1 μM for the positive control, acarbose, and were generally within 10–100 μM for xanthones: **1a** (50–100 μM); **1c** (20–50 μM), **2a** (50–100 μM), **2b** (20–60 μM), **2c** (10–40 μM), **3a** (10–40 μM); **3b** (10–30 μM), **3c** (10–40 μM) and γ-mangostin (**6**) (70–100 μM).

Briefly, in a 96-well plate, the enzyme (0.1 U mL^{-1}) dissolved in 100 mM phosphate buffer (pH 6.8) was incubated with the tested xanthones **1b–3c** at 37 °C for 10 min. After this period, the substrate CNPG3 was added (in final concentrations of 250, 500 and 1000 μM) and the enzymatic kinetics (37 °C) was monitored spectrophotometrically at 405 nm for 30 min. The obtained values correspond to the slope of the kinetic reaction, measured between 5 and 20 min, of at least three independent experiments.

The obtained results were fitted by nonlinear least squares regression to the generalized Michaelis–Menten model equation and to its corresponding simplifications, for the different types of inhibition, according to Rocha *et al.*[26] For each tested condition, the obtained values for the parameters of the simplest model (without inhibition) were used as primary values, proceeding with the following types of inhibition, competitive, noncompetitive, uncompetitive and mixed inhibition. To this, the Excel Microsoft Office™ with use of the Solver™ iterative optimization supplement was resorted according to the methodology proposed by Bezerra *et al.*[27] and Dias *et al.*[28] In addition, visual inspection of the model adequacy was made by graphing the reciprocal of the maximum velocity ($1/V_{max}$) (y axis) against the reciprocal of xanthone concentrations ($1/[S]$) (x axis), where V_{max} = maximum achievable velocity when 0.1 U mL^{-1} of enzyme is used; K_m = Michaelis–Menten constant in mM, K_{ic} = inhibitor dissociation constant of the enzyme inhibitor expressed in μM^{-1}, K_{iu} = inhibitor dissociation constant of enzyme–substrate–inhibitor complex expressed in μM^{-1}.[29,30]

One-way ANOVA analysis was performed to check for the procedure precision and inhibitory effect, considering a p value ≤0.05 statistically significant. In order to confirm the inhibition mechanism, the extra sum-of-squares F test[31] and the Akaike information criterion (AIC) test[32] provided a statistical comparison between models. The error value of the kinetic

constants was assessed by the Jackknife procedure, which involved the calculation of the standard deviation of all guesses made by Solver™ supplement when each experimental data point was removed from the initial set.

2.4. *In vitro* α-glucosidase inhibitory activity assay

The α-glucosidase inhibition assay was performed according to reported methods with slight modifications.[33] The assay was carried out by monitoring the α-glucosidase-mediated transformation of the substrate *p*NPG into α-D-glucose and α-nitrophenol, at 405 nm. Briefly, in a 96-well plate, the enzyme (0.05 U mL^{-1}) was dissolved in 100 mM phosphate buffer (pH 6.8) and incubated with the tested xanthones **1–6** (0–200 μM) at 37 °C for 5 min. Thereafter, the substrate *p*NPG (600 μM) was added and the enzymatic kinetics (37 °C) was monitored spectrophotometrically at 405 nm for 30 min. The obtained values correspond to the slope measured between 5 and 20 min of the kinetic reaction. The obtained results were expressed as the mean % inhibition ± SEM (eqn (1)) of α-glucosidase activity and represent at least three independent experiments. Acarbose (0–3000 μM) was used as the positive control.

2.5. Inhibitory kinetic analysis of α-glucosidase

Inhibitory kinetic analyses were performed for all tested xanthones, except for **1a** and **4** that showed residual activity at the highest tested concentrations of 50 and 200 μM, respectively. The tested concentrations were between 250–1500 μM for the positive control, acarbose, and generally within 5–130 μM for xanthones: **1b** (10–25 μM); **1c** and **3a** (5–20 μM); **2a** (10–30 μM), **2b**, **2c**, **3b** and **3c** (5–15 μM), α-mangostin **5** (120–130 μM) and γ-mangostin **6** (7–15 μM).

Briefly, in a 96-well plate, the enzyme (0.05 U mL^{-1}) dissolved in 100 mM phosphate buffer (pH 6.8) was incubated with the tested xanthones at 37 °C for 5 min. After this period, the substrate *p*NPG was added (in final concentrations of 300, 600 and 1200 μM) and the enzymatic kinetics was monitored spectrophotometrically at 405 nm for 30 min, at 37 °C. The obtained values correspond to the slope of the kinetic reaction, measured between 5 and 20 min, of at least three independent experiments.

The study of the inhibition type (competitive, uncompetitive, non-competitive or mixed) of the tested xanthones was performed using the nonlinear regression Michaelis–Menten enzyme kinetics and the corresponding Lineweaver–Burk double reciprocal plots for each concentration of the inhibitor and substrate. Additionally, the estimation of kinetic parameters and prediction of the actual mechanism of inhibition was performed by means of Microsoft Office Excel™ spreadsheets and using the Solver supplement add-in, as previously described for the α-amylase inhibitory kinetic analysis (section 2.3) (Table 2).[26]

2.6. Statistical analysis

The results of the *in vitro* inhibitory activities of the xanthones against pancreatic α-amylase and yeast α-glucosidase are expressed as mean ± SEM. A statistical comparison between the active xanthones was performed using one-way analysis of variance (ANOVA). Differences among the groups were compared by the Tukey test, with a p value ≤ 0.05 considered statistically significant. All the statistical analyses were performed using GraphPad Prism™ (version 5.0; GraphPad Software). The type of inhibition was established by comparison among the models using Solver™ and by applying the extra sum-of-square F test and AIC test. ANOVA was applied to evaluate the precision of the method.

3. Results

3.1. *In vitro* α-amylase activity

Table 1 lists the IC_{50} values for the inhibitory effect of xanthones **1–6** and for the positive control, acarbose, on the pancreatic α-amylase activity. The IC_{50} obtained for this control, 0.62 ± 0.07 μM, was significantly lower than those obtained for the studied xanthones **1–6** (Table 1 and Fig. 2). Nevertheless, inhibitory effects in a concentration-dependent manner (*e.g.* xanthone **3b** in Fig. 2), was noticed for all, except for derivative **1b** which was unable to reach a 40% effect, and mangiferin (**4**) which did not show any activity even at the highest tested concentrations (200 μM) (Table 1). Solubility constraints dictated the maximum of 90 μM for α-mangostin (**5**) that was possible to test, also with no inhibitory activity. Derivatives **2c**, **3a** and **3b** were the most efficient tested compounds, with $IC_{50} < 30$ μM. Derivatives **1a**, **1c** and **2a** were the less effective compounds, presenting IC_{50} values of 80 ± 4 μM, 66 ± 4 μM and 90 ± 3 μM, respectively (Table 1). In addition, γ-mangostin (**6**) presented an IC_{50} value of 103 ± 2 μM, almost 5-fold higher than the IC_{50} of the most active 2,3-diarylxanthone **3a** (Table 1) derivative.

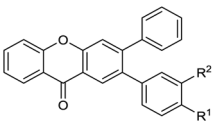
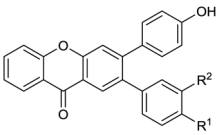
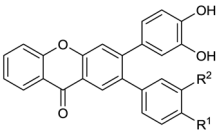
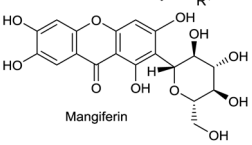
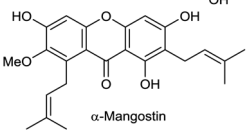
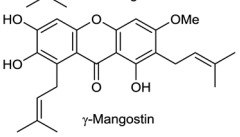
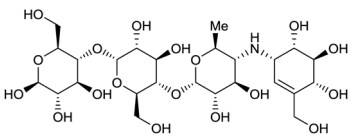
The type of α-amylase inhibition for all active xanthones and the positive control acarbose were determined from the statistical evaluations of the experimental data fitting to the corresponding Michaelis–Menten kinetics model and Lineweaver–Burk plots. The analysis by ANOVA one-factor of the experimental data showed a precision ranging from 1.0 and 2.8 Δabsorbance (ΔAbs) per min for the tested xanthones and 1.6 ΔAbs per min for acarbose, as calculated from the within-groups mean square.

The ANOVA also showed for the tested compounds F values of 25–143, higher than the F critical values (ranging from 1.95–2.01), corresponding to a p value <0.05 (one-tail probability), as a consequence of its significant effect on the enzymatic activity.

In all cases, the inhibition kinetic model was the best fitted, thus, providing the lowest sum squared residuals after iterative non-linear regression using the Solver™ supplement.

Table 2 depicts the considered inhibition mechanism and the respective kinetic constant values (V_{max}, K_m, K_{ic} and/or K_{iu}) for the studied xanthones. It indicates that xanthones **1a**, **1c**, **2a**, **2b** and **3a** act as non-competitive inhibitors of α-amylase activity, while compounds **2c**, **3b**, **3c** and γ-mangostin (**6**)

Table 1 Structures and inhibitory effects (IC_{50} μM, mean ± SEM) of the tested xanthones **1–6** on the porcine α-amylase and yeast α-glucosidase activities

Compounds		R^1	R^2	IC_{50} (μM) or Inhibition (%)	
				α-Amylase	α-Glucosidase
1a		H	H	80 ± 4	<20%$^{50\ \mu M\ a}$
1b		OH	H	<40%$^{200\ \mu M\ a}$	24 ± 1
1c		OH	OH	66 ± 4	13.5 ± 0.4
2a		H	H	90 ± 3	27.4 ± 0.9
2b		OH	H	35 ± 1	10.3 ± 0.2
2c		OH	OH	**27 ± 1**	**8.9 ± 0.3**
3a		H	H	**23 ± 1**	13 ± 2
3b		OH	H	**27 ± 1**	**8.6 ± 0.3**
3c		OH	OH	42 ± 1	**9.2 ± 0.4**
4	 Mangiferin			<20%$^{200\ \mu M\ a}$	<20%$^{200\ \mu M\ a}$
5	 α-Mangostin			<20%$^{90\ \mu M\ a}$	137 ± 2
6	 γ-Mangostin			103 ± 2	11.4 ± 0.3
Positive control Acarbose	***Positive control*** 			0.62 ± 0.07	515 ± 19

[a] The value represents the percentage of inhibition for the highest tested concentration (in superscript). Each study corresponds to at least three experiments.

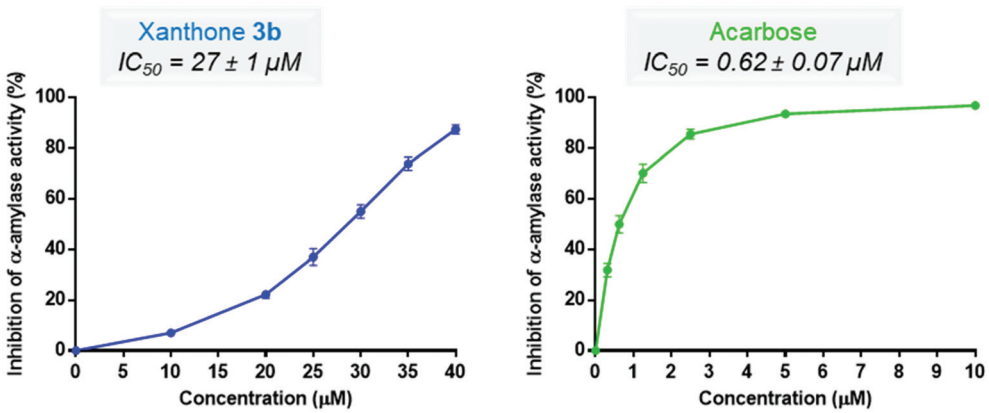


Fig. 2 α-Amylase inhibition by xanthone **3b** and the positive control, acarbose. Each value represents mean ± SEM of at least four experiments.

Table 2 Type of inhibition (using Solver™ supplement) of the tested xanthones and acarbose against α-amylase and α-glucosidase activities and respective kinetic parameter values: V_{max}, K_m, K_{ic} and K_{iu} (mean ± SEM)

Compounds	Type of inhibition	V_{max} (ΔAbs min^{-1})	K_m (μM)	K_{ic} (μM^{-1})	K_{iu} (μM^{-1})
α-Amylase activity					
1a	Non-competitive	42 ± 4	1084 ± 143	73 ± 6	73 ± 6
1c	Non-competitive	39 ± 1	957 ± 44	42 ± 1	42 ± 1
2a	Non-competitive	37 ± 2	1011 ± 71	91 ± 4	91 ± 4
2b	Non-competitive	38 ± 1	1009 ± 21	32.2 ± 0.3	32.2 ± 0.3
2c	Competitive	36.6 ± 0.4	908 ± 18	49 ± 1	—
3a	Non-competitive	42 ± 1	1009 ± 42	19 ± 1	19 ± 1
3b	Competitive	39 ± 2	1011 ± 78	28 ± 2	—
3c	Competitive	47 ± 2	1547 ± 58	82 ± 3	—
6	Competitive	41 ± 2	996 ± 73	138 ± 10	—
Acarbose	Mixed	41 ± 1	942 ± 53	2.6 ± 0.2	0.37 ± 0.01
α-Glucosidase activity					
1b	Non-competitive	120 ± 5	710 ± 59	21 ± 1	21 ± 1
1c	Non-competitive	115 ± 3	694 ± 33	20 ± 1	20 ± 1
2a	Non-competitive	131 ± 4	808 ± 35	38 ± 1	38 ± 1
2b	Non-competitive	121 ± 5	748 ± 50	12.9 ± 0.6	12.9 ± 0.6
2c	Non-competitive	116 ± 6	692 ± 62	9.6 ± 0.4	9.6 ± 0.4
3a	Non-competitive	125 ± 4	838 ± 42	21 ± 1	21 ± 1
3b	Non-competitive	143 ± 3	1025 ± 29	14.1 ± 0.2	14.1 ± 0.2
3c	Non-competitive	134 ± 6	950 ± 56	12.8 ± 0.4	12.8 ± 0.4
5	Non-competitive	78 ± 5	601 ± 85	166 ± 15	166 ± 15
6	Non-competitive	93 ± 6	781 ± 77	11.8 ± 0.6	11.8 ± 0.6
Acarbose	Competitive	125 ± 1	730 ± 13	312 ± 3	—

behaved as competitive inhibitors of α-amylase activity. As an example, the Lineweaver–Burk plots of α-amylase inhibition by the non-competitive inhibitor xanthone **2c**, and γ-mangostin (**6**) as the competitive inhibitor are shown in Fig. 3. Thus, on increasing the inhibitor concentration, the plots for non-competitive inhibitor **2c** showed constant K_m values with decreasing V_{max} values and for the competitive inhibitor γ-mangostin (**6**), the V_{max} value remained constant but with increasing K_m values.

The positive control acarbose exhibited a mixed type of inhibition, where the values of the kinetic parameters V_{max} and K_m decreased with increasing concentrations of acarbose.

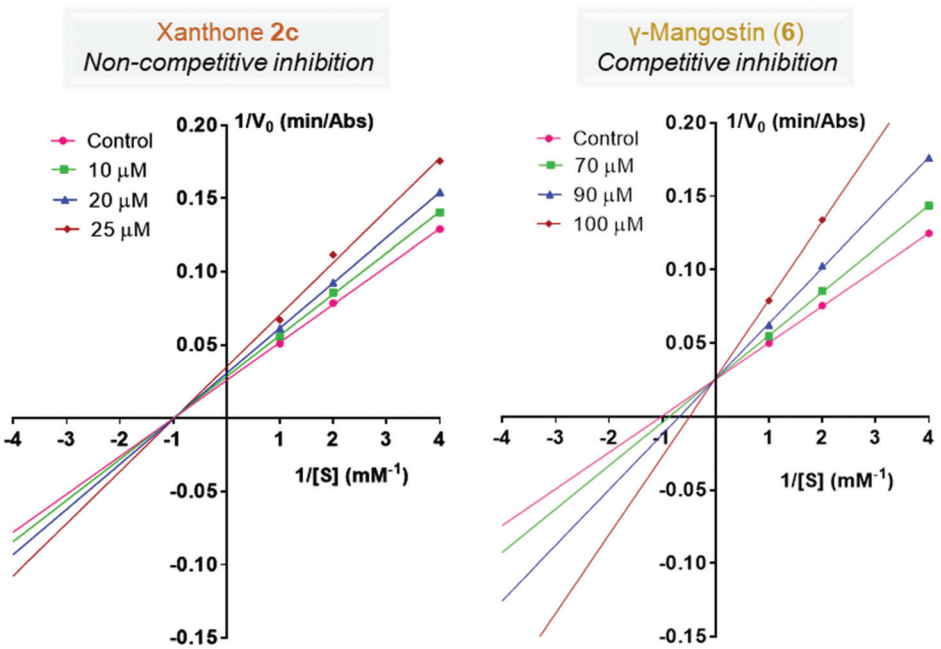


Fig. 3 Lineweaver–Burk plots of α-amylase inhibition by xanthone **2c** and γ-mangostin (**6**).

3.2. *In vitro* α-glucosidase activity

The results presented in Table 1 show the inhibitory activity of yeast α-glucosidase by xanthones **1–6**, and the positive control, acarbose.

The tested xanthones **1–6** proved to be effective inhibitors of α-glucosidase in a concentration-dependent manner (*e.g.* xanthone **3b** in Fig. 4), except for compound **1a**, which revealed no activity at the highest tested concentration (50 μM, due to solubility constraints) and mangiferin (**4**) which was also unable to inhibit the activity of the α-glucosidase enzyme at the maximum tested concentration of 200 μM. Xanthones **2c**, **3b** and **3c** were the best inhibitors and exhibited IC_{50} values of 8.9 ± 0.3 μM, 8.6 ± 0.3 μM and 9.2 ± 0.4 μM, respectively. The less active α-glucosidase inhibitors were xanthones **1b** and **2a**, presenting IC_{50} values of 24 ± 1 μM and 27.4 ± 0.9 μM, respectively.

Unlike the abovementioned α-amylase inhibitory activity, α-mangostin (**5**) was able to inhibit α-glucosidase activity, although in a less efficient manner than the other tested xanthones, providing an IC_{50} value of 137 ± 2 μM. γ-Mangostin (**6**) was revealed to be one of the most active compounds against α-glucosidase activity, presenting an IC_{50} of 11.4 ± 0.3 μM (Table 1).

The IC_{50} values presented in Table 1 for the α-glucosidase inhibitory activity vary from 8.6 up to 137 μM and are significantly lower than the IC_{50} value found for the positive control, acarbose (IC_{50} = 515 ± 19 μM) (Fig. 4).

The type of α-glucosidase inhibition for all active xanthones and the positive control acarbose were deduced from the statistical evaluations of the experimental data fitting to the corresponding Michaelis–Menten kinetics model and Lineweaver–Burk plots. The analysis by ANOVA one-factor of the experimental data showed a precision ranging from 3.0 and 7.3 ΔAbs per min for the tested xanthones and 2.9 ΔAbs per min for acarbose, as calculated from the within-groups mean square. In addition, a significant effect on the enzymatic activity by all active xanthones was also demonstrated since F values of 25–143 were higher than the F critical values (ranging from 1.95–2.04), corresponding to a p value <0.05 (one-tail probability).

In Table 2 are summarized the results for the type of inhibition against α-glucosidase and the kinetic constant values (V_{max}, K_m, K_{ic} and/or K_{iu}) for the studied xanthones, obtained from the nonlinear regression of respective inhibition theoretical models. All xanthones showed a non-competitive type of inhibition on the α-glucosidase activity. Fig. 5 shows an example of a Lineweaver–Burk plot from the xanthone **3a**. Thus, on increasing the concentration of compound **3a**, the K_m value remained constant but decreased V_{max} values were observed.

The positive control acarbose exhibited a competitive type of inhibition, where the K_m value increased and V_{max} value remained constant, with increasing concentrations of acarbose (Fig. 5).

4. Discussion

The number of studies involving xanthones as inhibitors of the α-amylase enzymatic activity is small and most are based on natural derivatives isolated from plants of Mangifera or Garcinia genus.[34,35] As far as is known, this is the first report on the α-amylase inhibitory activity of a range of new synthetic xanthones, which includes nine 2,3-diarylxanthone derivatives, with different substitution patterns (Fig. 1). Thus, compounds are numbered **1–3a–c**, in which compound **1a** is not substituted at rings D and E, compound **1b** is substituted with a *p*-hydroxy group at the D-ring and compound **1c** is substituted with a 3,4-dihydroxy group (catechol moiety). Compounds **2a–c** are substituted at the E-ring with a *p*-hydroxy group and maintaining the same substitutions at the D-ring mentioned above. For compounds **3a–c**, the E-ring is substituted with a catechol moiety keeping the same substitutions at the D-ring. This study was also applied to three natural xanthones obtained from commercial sources and therefore the results obtained were compared with the available information. Mangiferin (**4**),

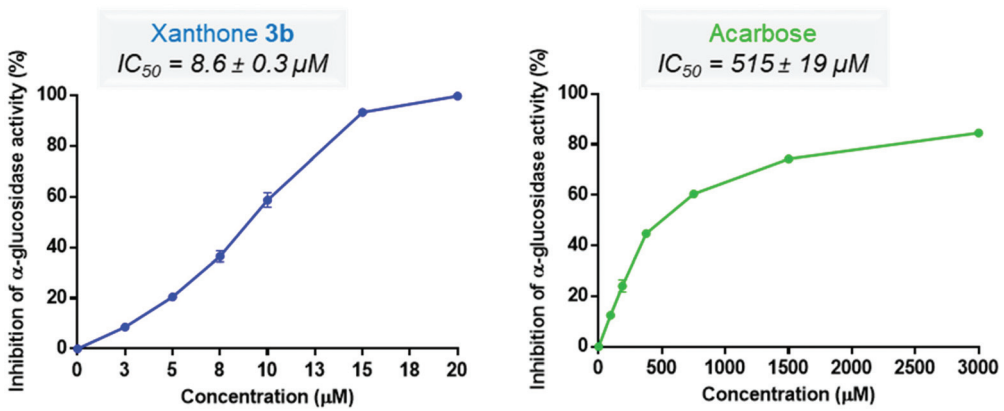


Fig. 4 α-Glucosidase inhibition by xanthone **3b** and the positive control, acarbose. Each value represents mean ± SEM of at least four experiments.

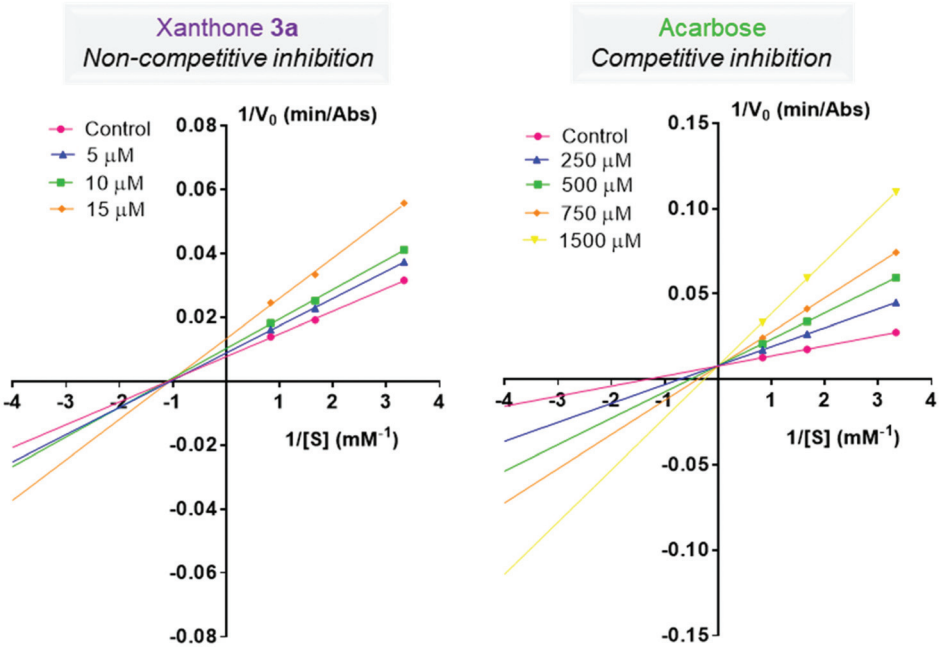


Fig. 5 Lineweaver–Burk plots of α-glucosidase inhibition by xanthone **3a** and the positive control acarbose.

which is the most abundant natural *C*-glycosylated xanthone, consists of 1,3,6,7-tetrahydroxy-9*H*-xanthen-9-one having a β-D-glucopyranosyl residue at C-2; α-mangostin (**5**), a tetra-oxygenated diprenylated xanthone, has the chemical structure of 1,3,6-trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-en-1-yl)-9*H*-xanthen-9-one and γ-mangostin (**6**), the methylated isomer of α-mangostin (**5**), has the chemical structure of 1,3,6,7-tetrahydroxy-2,8-bis(3-methylbut-2-en-1-yl)-9*H*-xanthen-9-one.

In accordance with the obtained results, the absence (xanthone **1a**) or the presence of a single hydroxy group (xanthones **1b** and **2a**) is not favorable for an inhibitory effect on the α-amylase activity. The most active compounds were xanthones **2c**, **3a** and **3b**, with inhibitory order potency as xanthone **3a** (IC_{50} = 23 ± 1 μM) > xanthone **2c** = xanthone **3b** (IC_{50} = 27 ± 1 μM). We can postulate about the positive effect the presence of catechol moiety at rings D or E brings, being slightly more important at ring E, on the basis of IC_{50} values obtained for the derivative **3a** and derivative **1c** (possessing a catechol moiety at ring D), of 66 ± 4 μM. Interestingly, the presence of two catechol moieties simultaneously at rings D and E did not improve the inhibitory effect on the α-amylase activity, as observed on comparing xanthone **3c** with xanthone **3a**. These observations seem to corroborate studies on the inhibiting effect of flavonoids, namely on the importance of hydroxy groups, as a catechol moiety at the B-ring, for the α-amylase inhibitory activity.[25,36]

The effects of three commercial xanthones [mangiferin (**4**), α-mangostin (**5**) and γ-mangostin (**6**)] against α-amylase were further considered in this work, since they are rare examples of xanthones that have been already studied by other authors, when isolated from natural sources. Thus, mangiferin (**4**), obtained from hydroalcoholic extracts of *Mangifera indica*,[34,37,38] was tested as an α-amylase inhibitor. On the other hand, Adnyana *et al.* isolated a parent xanthone and α-mangostin (**5**) from ethanol : water (50 : 50) extract of *Garcinia mangostana* pericarps and evaluated their α-amylase inhibitory profile.[35] Ibrahim *et al.* studied the properties of α-amylase inhibition by five xanthones isolated from the methanol : water (90 : 10) extract of *G. mangostana* pericarps (α-mangostin (**5**), β-mangostin, rubraxanthone, garcinone E and garcixanthone D),[39] five other xanthones from the methanol : water (70 : 30) extract of *G. mangostana* pericarps (garcixanthone A, gartanin, normangostin, garcinone C and garcimangostin A)[40] and also five xanthones isolated from the acetone extract of *G. mangostana* pericarps (γ-mangostin (**6**) and mangostanaxanthones I, II, VII and VIII).[41]

As expected, no activity was found for mangiferin (**4**) at the highest tested concentration of 200 μM. The single work on the inhibition of pancreatic α-amylase by mangiferin (**4**) was developed by Ganogpichayagrai *et al.* and revealed an IC_{50} of 1.0485 mg mL^{-1} (2.48 mM), considerably higher than the IC_{50} found in our tested experimental conditions.[37] The low solubility of trihydroxyxanthone α-mangostin (**5**) was limited to 90 μM, the highest concentration tested for this compound, and it showed no activity. In 2016, Adnyana *et al.* reported the inhibitory activity of unsubstituted xanthone and α-mangostin (**5**) against pancreatic α-amylase.[35] Both xanthones inhibited the enzyme in a concentration-dependent manner. The IC_{50} = 517.85 ± 8.52 μg mL^{-1} for the unsubstituted xanthone and the IC_{50} = 29.67 ± 1.98 μg mL^{-1} for α-mangostin (**5**) were higher than the positive control acarbose (IC_{50} = 14.33 ± 1.24 μg mL^{-1}).

γ-Mangostin (**6**), the demethylated isomer of α-mangostin (**5**), was revealed to be a poor inhibitor of the α-amylase enzymatic activity (IC_{50} = 103 ± 2 μM) when compared with the positive control acarbose (IC_{50} = 0.62 ± 0.07 μM), however, it is clearly more efficient than α-mangostin (**5**). In fact, γ-mangostin (**6**) reached 44% of inhibition for 100 μM concentration (Fig. 2) and no effect was registered for α-mangostin (**5**) at the maximum concentration of 90 μM.

Nevertheless, the positive control, acarbose, was noticeably more effective than the tested xanthones.

The type of α-amylase inhibition and the kinetic parameters of all active compounds and the positive control acarbose were determined here for the first time, using Lineweaver–Burk plots and the solver supplement of Excel Microsoft Office™. Both strategies were in accordance and a competitive type of inhibition for compounds **2c**, **3b**, **3c** and γ-mangostin (**6**) was observed. This competitive mechanism is implicated when the inhibitor competes directly with the substrate for the active site of the enzyme, requiring a higher concentration of substrate to generate the reaction product in the same period of time. Xanthones **1a**, **1c**, **2a**, **2b** and **3a** exhibited a non-competitive type of inhibition on α-amylase activity, meaning that, xanthones share the same affinity for both enzyme and enzyme–substrate complex, leading to a decrease of the enzymatic activity that cannot be overcome by increasing the substrate concentration.

Acarbose was identified as a mixed inhibitor, a similar behavior previously described by other authors for the inhibition of pancreatic α-amylase enzymatic activity.[25,42]

An important strategy to control blood sugar levels that will be absorbed in the small intestine and therefore, in the management of Type 2 DM also includes the inhibition of α-glucosidase enzyme involved in the carbohydrate digestion.[43] Unlike α-amylase, several studies involving either natural or synthetic xanthones as α-glucosidase inhibitors have been reported in the last two decades and reviewed by us in 2018.[21] A wide variety of natural derivatives with different substitution patterns (hydroxy, methoxy and prenyl are the most frequent ones) have been isolated, mainly from Swertia and Garcinia genera, and identified with a promising α-glucosidase inhibitory profile. For the synthetic derivatives, various alkyl and aryl substituents have been introduced in the xanthone core and some of them led to a high inhibition of α-glucosidase activity as well.

Concerning our results on 2,3-diarylxanthones **1–3a–c**, 2,3-diphenylxantone **1a**, and the absence of hydroxy groups at the D- or E-ring, rendered ineffective compounds up to the highest tested concentration (50 μM). Compounds **1b** (IC_{50} = 24 ± 1 μM) and **2a** (IC_{50} = 27.4 ± 0.9 μM), both bearing a single hydroxy group at the D- or E-ring, respectively, presented similar and the weakest inhibitory behavior among the 2,3-diarylxanthones tested. The introduction of an extra *m*-hydroxy group to form the catechol unit led to an increase of the inhibitory activity and with a similar effect, as can be observed by comparison of the IC_{50} values of xanthone **1c** (IC_{50} = 13.5 ± 0.4 μM) and xanthone **3a** (IC_{50} = 13 ± 2 μM). Moreover, the presence of a catechol moiety at the D- or E-ring and an extra *p*-hydroxy group at the E- and D-ring, respectively, contributed to the highest inhibitory activity of the hydroxylated 2,3-diarylxanthones **1–3** and with similar efficacy, for xanthone **2c** (IC_{50} = 8.9 ± 0.3 μM) and xanthone **3b** (IC_{50} = 8.6 ± 0.3 μM).

The presence of two catechol moieties, respectively at the D- and E-ring, did not improve the α-glucosidase inhibitory activity, as concluded by comparing the IC_{50} value of xanthone **3c** (IC_{50} = 9.2 ± 0.4 μM) with those of xanthones **2c** and **3b**. Interestingly, all hydroxylated xanthones **1–3** were significantly more active (with IC_{50} values varying from 8.6 to 137 μM) than the positive control acarbose (IC_{50} = 515 ± 19 μM). These results are in accordance with Liu *et al.* who synthesized and evaluated the α-glucosidase inhibitory activity of thirty xanthones possessing hydroxy substituents, as well as their acetoxy and alkoxy derivatives.[22] According to their results, polyhydroxyxanthones exhibited higher inhibitory activities than the corresponding acetoxy- and alkoxy-derivatives and that the number of hydroxy groups in the xanthone core was crucial for the inhibitory activity, with an order of potency of: tetrahydroxy > trihydroxy > dihydroxy > monohydroxy derivatives.

The effects of the commercial xanthones mangiferin (**4**), α-mangostin (**5**) and γ-mangostin (**6**) against α-glucosidase were also tested. In our study, no activity was found for mangiferin (**4**) at the maximum concentration of 200 μM. The literature concerning the effects of mangiferin (**4**) against yeast α-glucosidase activity is very controversial. Sekar *et al.* reported a rare example in which mangiferin (**4**) (IC_{50} = 36.84 μg mL^{-1} ≈ 87.23 μM) is less active than the acarbose (IC_{50} = 21.33 μg mL^{-1} ≈ 33.04 μM).[34] Other studies revealed that mangiferin (**4**) is more active than the positive controls used. As an example, Vo *et al.*[44] reported an IC_{50} value of 5.82 μg mL^{-1} ≈ 13.78 μM (acarbose, IC_{50} = 199.47 μg mL^{-1}, ≈309 μM) and Shi *et al.*[45] reported an IC_{50} value of 358.54 μM (acarbose, IC_{50} = 479.2 μM) for mangiferin (**4**), while other authors reported IC_{50} values in the mM range.[37,46] To complement this information, studies conducted on mangiferin (**4**) against both α-amylase and α-glucosidase enzymes concluded that the xanthone had more potential to inhibit α-glucosidase than α-amylase as reported by Ganogpichayagrai *et al.* (IC_{50} = 0.5813 mg mL^{-1} ≈ 1.38 mM and 1.0485 mg mL^{-1} ≈ 2.48 mM, respectively)[37] and by Dineshkumar *et al.* (IC_{50} = 41.88 μg mL^{-1} ≈ 99.2 μM and 74.35 μg mL^{-1} ≈ 176.0 μM, respectively).[38]

Unlike the α-amylase inhibitory assay, α-mangostin (**5**) was able to inhibit α-glucosidase activity presenting an IC_{50} value of 137 ± 2 μM, a higher effect than that recorded for the positive control acarbose (IC_{50} = 515 ± 19 μM) but weaker when compared to other hydroxylated xanthones. Here again, the literature provides contradicting data. Trinh *et al.*[47] reported a weak activity for α-mangostin (**5**) while Nguyen *et al.*[48] obtained an IC_{50} value of 11.4 μM (acarbose, IC_{50} = 214.5 μM), Phukhatmuen *et al.*[49] obtained an IC_{50} value of 15 μM (acarbose, IC_{50} = 93.3 μM), and Vongsak *et al.*[50] obtained an IC_{50} value of 29.27 μM (acarbose, IC_{50} = 241.36 μM).

γ-Mangostin (**6**) proved to be an effective inhibitor of α-glucosidase activity (IC_{50} = 11.4 ± 0.3 μM) in comparison with the results obtained for the positive control acarbose (IC_{50} = 515 ± 19 μM). These results were consistent with those developed by Trinh *et al.*[47] on γ-mangostin (**6**) where they reported an IC_{50} value of 15.3 ± 0.9 μM and those by Vongsak *et al.*[50] where they registered an IC_{50} value of 4.22 ± 0.58 μM.

It is known that a strong inhibition of α-amylase together with a mild inhibition of α-glucosidase results in an accumulation of undigested carbohydrates in the intestine, resulting in gastrointestinal side effects. Interestingly, the panel of xanthones assessed herein revealed that the most active compounds for α-glucosidase activity, exhibited only a moderate inhibition of α-amylase, with at least two fold high IC_{50} values relative to the ones found for α-glucosidase.

Regarding the mechanism of inhibition on α-glucosidase, all the active xanthones presented a non-competitive type of inhibition, indicating that these compounds bind at an allosteric site, lowering the catalytic efficiency of the enzyme. Other authors described that mangiferin (**4**) inhibited yeast α-glucosidase also in a non-competitive manner[45] while α-mangostin (**5**) and γ-mangostin (**6**) showed a mixed type of inhibition.[51]

As previously described by some authors and corroborating our results, acarbose presented a competitive type of inhibition.[25,36,45]

4. Conclusions

In this study, a series of xanthone derivatives were evaluated against α-amylase and α-glucosidase inhibitory activity and compared with the standard inhibitor, acarbose. Some of the tested xanthones were shown to be effective inhibitors of both enzymes, while exhibiting a stronger inhibition against α-glucosidase. It was possible to conclude that the number and position of the substituents determined the inhibitory profile of the studied compounds. Generally, the 2,3-diarylxanthones **1–3** and γ-mangostin (**6**) were more active against α-glucosidase than for α-amylase and displayed higher inhibitory activities against α-glucosidase than acarbose. Moreover, 2,3-diarylxanthones **2c**, **3b** and **3c** and γ-mangostin (**6**) showed a competitive type of inhibition for α-amylase while all hydroxylated xanthones acted *via* a non-competitive inhibition mechanism on α-glucosidase.

In conclusion, the inhibitory profile demonstrated by a series of xanthones against both α-amylase and α-glucosidase, and the established SAR, can be a good indicator of the potential of this family of compounds as a new therapeutic option in the management of type 2 DM.

Author contributions

Conceptualization, C. M. M. S., A. M. S. S. and E. F.; writing – original draft preparation, C. M. M. S., M. F.; writing – review and editing, C. M. M. S., C. P., M. F., A. N. A., A. M. S. S. and E. F.; supervision, M. F. and E. F. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts of interest to declare by the authors.

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