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Potential anti-diabetic properties of Merlot grape pomace extract: An *in vitro*, *in silico* and *in vivo* study of α-amylase and α-glucosidase inhibition

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ABSTRACT

A practical approach to control glycemia in diabetes is to use plant natural products that delay hydrolysis of complex sugars and promote the diminution of the release of glucosyl units into the blood plasma. Polyphenolics have been described as being effective in inhibiting amylases and α-glucosidases. Grape pomace is an important sub product of the wine industry, still rich in many compounds such as polyphenolics. In this context, the purpose of this study was to search for possible effects of a grape pomace extract on salivary and pancreatic α-amylases and α-glucosidase, as well as on intestinal glucose absorption. The Merlot grape pomace extract (MGPE) was prepared using a hydroalcoholic mixture (40% ethanol + 60% water). *In vitro* inhibition was quantified using potato starch (for amylases) and maltose (for α-glucosidase) as substrates. *In vivo* inhibition was evaluated by running starch and maltose tolerance tests in rats with or without administration of MGPE. Ranking of the extract compounds for its affinity to the α-amylases was accomplished by computer simulations using three different programs. Both α-amylases, pancreatic and salivary, were inhibited by the MGPE. No inhibition on α-glucosidase, however, was detected. The IC_{50} values were 90 ± 10 μg/mL and 143 ± 15 μg/mL for salivary and pancreatic amylases, respectively. Kinetically this inhibition showed a complex pattern, with multiple binding of the extract constituents to the enzymes. Furthermore, the *in silico* docking simulations indicated that several phenolic substances, e.g., peonidin-3-*O*-acetylglucoside, quercetin-3-*O*-glucuronide and isorhamnetin-3-*O*-glucoside, besides catechin, were the most likely polyphenols responsible for the α-amylase inhibition caused by MGPE. The hyperglycemic burst, an usual phenomenon that follows starch administration, was substantially inhibited by the MGPE. Our results suggest that the MGPE can be adequate for maintaining normal blood levels after food ingestion.

1. Introduction

Diabetes is a well-known chronic disease that causes hyperglycemia and modifies metabolism in a disordered way. The prevalence of diabetes mellitus steadily increases worldwide and has far-reaching consequences for the quality of human life. Diabetes mellitus of type 2 (DM2) affects about 90% of patients diagnosed with the disease in high-income countries (Ma et al., 2019). Hyperglycemia, the most visible consequence of diabetes, originates several metabolic disturbances (Giovannini, Howes, & Edwards, 2016). Many oral antidiabetic drugs are used for controlling glycemia. These antidiabetics can be divided in various classes according to their mechanisms of action: (1) insulin secretagogues (e.g., sulphonylureas and postprandial glucose regulators), (2) compounds able to reduce insulin resistance (e.g., metformin and thiazolidinediones), (3) carbohydrate digestive enzymes inhibitors (e.g., acarbose) (Campbell, 2007). The use of these drugs may

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present several degrees of efficiency, depending on each individual case. Several of them act by means of various mechanisms, as for example the soy isoflavones, which behave as insulin secretagoges and at the same time are inhibitors of carbohydrate digestive enzymes (Hamden, Jaouadi, Carreau, Aouidet, & Elfeki, 2011). On the other hand, their use is not free of several side effects. The latter include hypoglycemia, weight gain, edemas, lactic acidosis, cholestasis, folate and B_{12} malabsorption, etc. Acarbose, which acts by inhibiting α-glucosidase and α-amylase (respectively, EC. 3.2.1.20 and EC. 3.2.1.1), causes abdominal discomfort, flatulence, meteorism and moderate diarrhea. These may be the result of increased and abnormal fermentation of semi digested carbohydrates in consequence of the action of the gut microbiota (Sales, Souza, Simeoni, Magalhães, & Dâmaris, 2012). Such undesired effects very often cause discontinuation of therapy. For this reason, the introduction of new inhibitors is highly desirable.

In spite of the negative side-effects of acarbose, retardation of glucose absorption during digestion has been widely considered a useful and practical manoeuvre for controlling blood glucose concentration (Lalegani, Gavlighi, Azizi, & Sarteshnizi, 2018). This is one more reason for the present outstanding interest in plant-derived medicines and food additives containing mixtures of inhibitors capable of inhibiting carbohydrate hydrolysing enzymes without causing the side effects of acarbose. The idea is based on the hope that, if ingested as food or during the meals in conjunction with food, their mild and paused action may prevent accumulation of undigested carbohydrates. This, in turn, would reduce the risk of excessive bacterial fermentation. Polyphenolics, including tannins of various types, flavonoids, catechins and gallic acid among others, have been denoted as antioxidants and inhibitors of hydrolytic enzymes, especially of α-amylases and α-glucosidase (Silva et al., 2014; Martinez-Gonzalez et al., 2017; Kato et al., 2017; Kato-Schwartz et al., 2018; Shanmugam et al., 2018; Gutiérrez-Grijalva, Antunes-Ricardo, Acosta-Estrada, Gutiérrez-Uribe, & Heredia, 2019; Li, Wang, et al., 2019; Cardullo et al., 2020; Zhu, Chen, Zhang, & Huang, 2020).

Among grapes, *Vitis vinifera* is one of the most frequently cultivated species for wine production (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Dulf, Vodnar, Toşa, & Dulf, 2020). The vitiviniculture branch of activities generates many side-products, e.g., lees, rachis and pomace. When their quantities are summed up, they correspond to approximately 30% of the vinified grapes (Drevelegka & Goula, 2020). This material is still underexplored, and it is frequently discarded into the environment, regardless of its negative impact (Beres et al., 2017). Grape pomace, the most massive sub-product, comprises stems, skins and seeds. As it is easy to deduce, this material still contains appreciable amounts of phenolic compounds and other active principles (Corrêa et al., 2017; Peixoto et al., 2018).

The phenolic compounds have been claimed to possess antioxidant, antiradical, antitumor, anti-microbial, anti-aging, anti-inflammatory and anti-tumoral properties and also of being able to exert cardioprotective activity (Corrêa et al., 2018; Jara-Palacios et al., 2015; Ribeiro et al., 2015; Zhao, Zhang, Zhang, He, & Duan, 2020). Previous investigations of our group, for instance, have identified several molecules with potential functional properties in a Merlot (*Vitis vinifera* L.) grape pomace hydroalcoholic extract (MGPE). The described compounds include phenolic non-anthocyanins, such as various catechins, as well as phenolic anthocyanins (Corrêa et al., 2017; Gonçalves et al., 2017) (Table 1). The studied MGPE underwents more or less drastic qualitative and quantitative modifications in its phenolic contents upon digestion, as indicated by an *in vitro* digestion study (Corrêa et al., 2017). Nonetheless, it is still markedly able to ameliorate the oxidative state and to mitigate inflammation of arthritic rats (Gonçalves et al., 2017).

Recently, extracts of several Brazilian native fruits such as *Campomanesia phaea, Eugenia dysenterica*, *Myrciaria dubia* and *Myrciaria cauliflora* have been reported to effectively inhibit α-amylases in *in vitro* and *in vivo* (animal experiments and clinical studies) (Balisteiro, de Araujo, Giacaglia, & Genovese, 2017). Similar results have been obtained with extracts of red rice (*Oryza sativa*) (Liu et al., 2017), fig leaves (*Ficus benjamina*) (Mumtaz et al., 2018), and apple fruits (*Malus domestica*) (Li, Sun, et al., 2019). The polyphenols have generally been considered the most important molecules involved in these effects (Sun, Warren, & Gidley, 2019; Tresserra-Rimbau, Lamuela-Raventos, & Moreno, 2018). On the other side, as long as we could find out, no attempts have been done to investigate possible inhibitory activities of grape pomace extracts on enzymes responsible for the hydrolysis of carbohydrates that are important in type 2 diabetes. Considering this gap, the purposes of the present work were to investigate the Merlot grape pomace extract for its actions on three carbohydrate-hydrolysing enzymes, more specifically salivary and pancreatic α-amylases and α-glucosidase, as well as to assess its actions on intestinal glucose absorption. It should be remarked that potential health benefits for the Merlot grape pomace also presents a sustainable alternative for its use. Once an inhibitory effect has been detected, this primary observation can be complemented by experiments aiming at understanding the binding interactions involved in the inhibition.

Table 1
Daily doses of phenolic compounds, non-anthocyanins and anthocyanins, administered to the animals*.

Compound	Daily dose of each compound (mg/kg)	
	50 mg/kg dose	250 mg/kg dose
Non-anthocyanin phenolic compounds		
Galloylshikimic acid	0.17	0.84
B-type (epi)catechin dimers (4 isomers)	1.28	6.36
Digalloylquinic acid	0.12	0.58
(+)-Catechin	0.36	1.82
Digalloylshikimic acid	0.09	0.47
(−)-Epicatechin	0.37	1.83
B-type (epi)catechin trimers (2 isomers)	0.43	2.13
B-type (epi)catechin tretramer	0.16	0.8
Myricetin-*O*-hexoside	0.07	0.36
Quercetin-3-*O*-glucuronide	0.03	0.14
Quercetin-3-*O*-glucoside	0.03	0.13
Laricitrin-*O*-hexoside	0.02	0.09
Quercetin-*O*-pentoside	0.02	0.10
Quercetin-*O*-rhamnoside	0.02	0.10
Isorhamnetin-3-*O*-glucoside	0.03	0.13
Methylisorhamnetin derivative	0.02	0.08
Anthocyanins		
Petunidin-3-*O*-glucoside	0.03	0.15
Peonidin-3-*O*-glucoside	0.08	0.39
Malvidin-3-*O*-glucoside	0.17	0.85
Peonidin-3-*O*-acetylglucoside	0.04	0.17
Malvidin-3-*O*-acetylglucoside	0.04	0.19

*Values were calculated from the corresponding contents of the grape pomace aqueous extract reported in a previous work (Corrêa et al., 2017).

2. Material and methods

2.1. Materials

Enzymes and most specific chemicals were obtained from Sigma-Aldrich (St. Louis, USA): pancreatic α-amylase (porcine; Type VI-B; A3176), salivary α-amylase (human; A1031), maltose (M5885), glucose (G8270), potato starch (85642) and acarbose ($C_{25}H_{43}NO_{18}$, mw 645; A8980). Gold Analisa was purchased from Labtest Brazil. Samples of Merlot grape pomace (*Vitis vinifera*) were obtained from companies established in the Paraná State, Brazil. The grapes had been harvested and pressed in 2013. In the laboratory, drying was done in a convection oven (Marconi MA 035, Brazil) at 80 °C for 36 h. After milling, the material was packed in polyethylene film bags under vacuum with subsequent storing at −20 °C until use (Ribeiro et al., 2015).

2.2. Merlot grape pomace extract preparation

The Merlot grape pomace extract (MGPE) was prepared as described before (Gonçalves et al., 2017). A full characterization of the extract in terms of its non-anthocyanins and anthocyanin constituents by HPLC-DAD-MS analysis has already been reported (Gonçalves et al., 2017). Names and amounts of the identified compounds are given in Table 1. In this list the B-type (epi)catechin dimers (4 isomers) and B-type (epi) catechin trimers (2 isomers) were added together.

2.3. Initial reaction rates of α-amylases

Initial reaction rates catalyzed by both α-amylases, human salivary and porcine pancreatic, were measured at temperature (37 °C) and pH (6.9) close to the optima reported previously (Silva et al., 2014). The substrate was potato starch up to 1.0 g per 100 mL prepared in 20 mmol/L phosphate buffer plus 6.7 mmol/L NaCl. The amount of enzyme added to each reaction mixture was 1 unit (specific activity 500 units/mg protein). After 10 min reaction, the amount of reducing sugars in the reaction medium was quantified using the 3,5-dinitrosalicylic acid procedure (Miller, 1959). The standard curve was constructed with maltose. No changes in pH were observed along the incubation time. Up to 250 μg/mL MGPE concentrations were used in the various assays.

2.4. Isolation and assay of the intestinal α-glucosidase

The intestinal α-glucosidase (EC. 3.2.1.20) was isolated from the small intestine of the rat as described previously (Kato-Schwartz et al., 2020). For the α-glucosidase activity determination a glucose diagnosis kit based on the glucose oxidase reagent was used as described (Kato-Schwartz et al., 2020). The enzyme unit definition was based on the universally accepted rules.

2.5. Kinetic evaluation of the inhibitory action of MGPE on the α-amylases

The inhibitor concentrations causing 50% inhibition (IC_{50}) were obtained by numerical interpolation using the Scientist program (MicroMath Scientific Software (Salt Lake City, UT). Fitting of the rate equations to the experimental initial rates of enzyme activity was done using the traditional non-linear least-squares algorithm. The most adequate equation was inferred from the standard deviations and model selection criteria (MSC). The model selection criterion is usually defined as (Akaike, 1974):

$$MSC = \ln\left[\frac{\sum_{i=1}^{n} w_i\left(Y_{obs_i} - \bar{Y}_{obs}\right)^2}{\sum_{i=1}^{n} w_i\left(Y_{obs_i} - Y_{cal_i}\right)^2}\right] - \frac{2p}{n} \quad (1)$$

Y_{obs}, $\bar{Y}_{obs}$ and Y_{cal} are, respectively, the experimental, the mean experimental and the calculated reaction rates. The number of observations is represented by n and the number of parameters by p. The kinetic mechanism with the largest MSC value was taken as the best description of the experimental data.

2.6. Bioinformatics

The 3D structure of pancreatic α-amylase from pig at 1.38 Å resolution bonded to acarbose fragment (pseudotrisaccharide), was taken from the Protein Data Bank (PDBid 1hx0) [PMID 11412124]. Next, the human pancreatic α-amylase bonded to acarbose pentasacharide (ARE) (PDBid 1xd0) (Li et al., 2005), was obtained and superimposed on the porcine pancreatic α-amylase using Coot software (Emsley, Lohkamp, Scott, & Cowtan, 2010). The coordinates of the ARE ligand was then transfered to the pancreatic α-amylase (geometric docking) so the resulting structure complex was used as a main target in virtual screening simulations. Human salivary α-amylase (1.5 Å resolution; pdbid: 3dhp) was used as secondary target in the docking studies to evaluate the best compounds selected by virtual screening. All solvent molecules (water) and ions were eliminated from the structures before proceeding to the docking and virtual screening.

Three programs were used in the simulations: Autodock-v4.2.3 (Morris et al., 2009), implemented in the PyRx-0.9 graphical interface (Dallakyan & Olson, 2015), Molegro-v6.0 Virtual Docker (Thomsen & Christensen, 2006) and Gold-v5.4.0 (Jones, Willett, Glen, Leach, & Taylor, 1997). The choice for search and scoring functions in each program was accomplished through redocking of the ARE ligand onto the previously modelled porcine pancreatic α-amylase complex. The protocol was considered to be validated when the best ranked pose provided a root mean-square deviation below 2.0 Å in all repetitions. For Molegro, the Moldock Score [grid] and Iterated Simplex were selected as scoring and search algorithms, respectively. The search was adjusted to 10 runs, while other parameters were set as default. A search radius of 12 Å centralized at the reference ligand (ARE) was used. For Autodock, the search box was centered at the ligand, with *x, y* and *z* axes having grid dimensions of 50. The number of runs was increased to 50 and the number of energy evaluations was set to 2,500,000 (medium). The latter is justified by the large number of rotatable bonds of the ligands. Finally, for Gold, the selected scoring function was Chemplp (with ASP as rescore), the search efficiency was adjusted to 200% and the binding site was defined as all atoms within 10 Å from the reference ligand.

A virtual library of compounds was built using the molecules identified by HPLC-DAD-MS in the MGPE (Gonçalves et al., 2017). Their tridimensional structures were obtained from PubChem (Kim et al., 2019) or Zinc15 (Sterling & Irwin, 2015) databases. The structures of 19 compounds were downloaded, including procyanidin B2, the [epicatechin-(4β→8)-epicatechin] dimer, which served as the basis for drawing the β-type (epi)catechin trimer and β-type (epi)catechin tetramer compounds using Marvin Sketch program. The final library containing 21 molecules was then, virtually screened using the validated protocols established by redocking.

2.7. Animal experiments

Male Wistar rats (200–250 g) were used in all experiments. During the whole experimental time the animals were kept in individual cages with free access to water and standard pelleted food. The experiments were preceded by a fasting period of 18 h. The experiments were approved by the Ethics Committee for Animal Experimentation of the University of Maringá (Protocol number 067/2014-CEUA-UEM), which obeys the universally recommended rules for animal experimentation and is in full accordance with the Brazilian regulations on the subject.

2.7.1. Evaluation of glucose in blood

Blood glucose concentration was measured at time zero and at various times following the administration of carbohydrates and inhibitors. The carbohydrates were starch, maltose and glucose. The inhibitors were acarbose and pomace extract. Blood was collected from the tail vein at times 0, 15, 30, 45, and 60 min. Glucose was quantified by means of an Accu-Chek® Active Glucose Meter.

2.7.2. Blood glucose concentration after starch administration

Intragastric administration was used throughout. The following experimental conditions were used: (1) controls, rats receiving corn starch (1 g per kg body weight); (2) negative controls, animals receiving tap water; (3) positive controls, 4 animals receiving corn starch plus acarbose (50 mg/kg); (4) test animals receiving corn starch plus 50 mg/kg MGPE; (5) test animals receiving corn starch plus 250 mg/kg MGPE. Four rats were used for each experimental protocol (n = 4). The decision on the doses of acarbose and MGPE was based on previous

experiments. Glucose concentration in blood was determined as described in item 2.7.1.

2.7.3. Blood glucose concentration after maltose administration

The following experimental conditions were used: (1) controls, rats receiving maltose (1 g per kg body weight); (2) negative controls, animals receiving tap water; (3) test animals receiving maltose plus 250 mg/kg MGPE; (4) test animals receiving maltose plus 500 mg/kg MGPE. Four rats were used for each experimental protocol (n = 4). Glucose concentration in blood was determined as described in item 2.7.1.

2.7.4. Glycemic levels after glucose feeding

The following experimental conditions were used: (1) controls, rats receiving glucose (1 g per kg body weight); (2) negative controls, animals receiving tap water; (3) test animals receiving glucose plus MGPE at the dose of 100 mg/kg; (4) test animals receiving glucose plus MGPE at the dose of 500 mg/kg. Four rats were used for each experimental protocol (n = 4). Glucose concentration in blood was determined as described in item 2.7.1.

2.8. Statistical analysis

The GraphPad Prism software (version 8.0) was used in the computations. Significant differences were inferred from the Tukey test for homogeneous data and from the Kruskal–Wallis test for heterogeneous data after one-way ANOVA. Significance was accepted when $p \leq 0.05$.

3. Results and discussion

3.1. In vitro inhibition of the activities of carbohydrate digestive enzymes by the grape pomace extract

The actions of the grape pomace extract on the activities of the various enzymes investigated in this work are shown in Fig. 1. The inhibitor concentration dependences were determined by fixing the starch concentration at 1 g/100 mL and by varying the concentrations of MGPE in the range up to 250 μg/mL. In Fig. 1A the residual enzymatic activities were plotted versus the inhibitor concentration. Both amylases were inhibited by the grape pomace extract with clear concentration dependences. Inhibition of the salivary amylase was more pronounced than that of the pancreatic amylase; the IC_{50} values were 90 ± 10 μg/mL for the first one and 143 ± 15 μg/mL for the latter. The effects of the MGPE on the intestinal α-glucosidase are also shown in Fig. 1A. In the pertinent experiments the concentration dependence was determined by fixing the maltose concentration at 25 mM and by varying the concentration of the grape pomace extract in the range up to 250 μg/mL. Clearly, no inhibition was observed ($p \geq 0.05$).

The representation of 1/v against the inhibitor concentration in Fig. 1B was done in order to infer in a preliminary way about the kinetic equation that describes the inhibition caused by the MGPE. With both enzymes a parabolic relationship was found, indicating that the rate equation must contain at least one quadratic [I] term (Plowman, 1972). It is worth mentioning that this phenomenon does not reflect the heterogeneity of the MGPE provided that the proportion between the various compounds is not changed (Plowman, 1972). With this preliminary notion in mind and for extending analysis, initial reaction rates were quantified by varying simultaneously the inhibitor and substrate concentrations. Fig. 2A displays the results using the human salivary α-amylase. Two different inhibitor concentrations were tested, as indicated on the graph. The usual saturation curves were found. The latter do not converge at the highest substrate concentrations, an observation that rules out competitive inhibition. In consequence, equations describing mixed inhibition were fitted to the experimental curves. Fitting was simultaneous, this includes the v × [S] relationship as well as the v × [I] relationship. The rate equation yielding the best

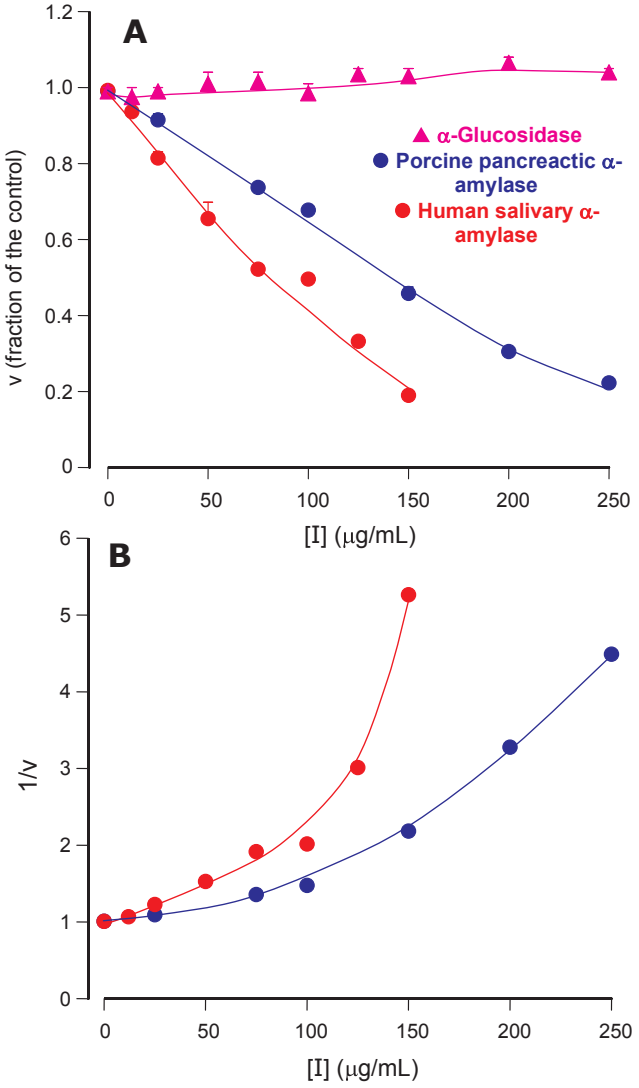

Fig. 1. Concentration dependences of the effects of the grape pomace extract on the activities of porcine pancreatic α-amylase, human salivary α-amylase and α-glucosidase. Each datum point is the mean of four determinations. Reaction rates (v; panel A) and reciprocals of the reaction rates (1/v; panel B) were represented against the grape pomace extract concentration ([I]).

fit to the experimental curves was one containing solely one quadratic term for [I]:

$$v = \frac{V_{\max}[S]}{K_M\left(1 + \frac{[I]}{K_{i1}} + \frac{[I]^2}{K_{i1}K_{i1}'}\right) + [S]\left(1 + \frac{[I]}{K_{i2}}\right)} \quad (2)$$

In Eq. (2) K_M and V_{max} represent, as usual, the Michaelis-Menten constants and the maximal reaction rate, respectively. The apparent inhibition constants are K_{i1}, K_{i1}' and K_{i2}, whose meaning is certainly very complex, but that bear relation to the true dissociation constants of complexes EI, EI_2 and ESI, respectively. The legend to Fig. 2 lists the optimized values of each parameter. The complex ESI_2 could not be detected, as the fitting procedure invariably returned K_{i2}' values exceeding by several orders of magnitude the other constants and with a standard deviation exceeding its optimized value by far. The lines running through the experimental points in both panels of Fig. 2 are the calculated curves. There is good agreement between theory and experiment, including the v × [I] relationship. If the optimized parameters displayed in the legend to Fig. 2 are substituted into equation (2), it will be possible to predict the reaction rates for any combination of starch and extract concentrations. Comparison of the optimized

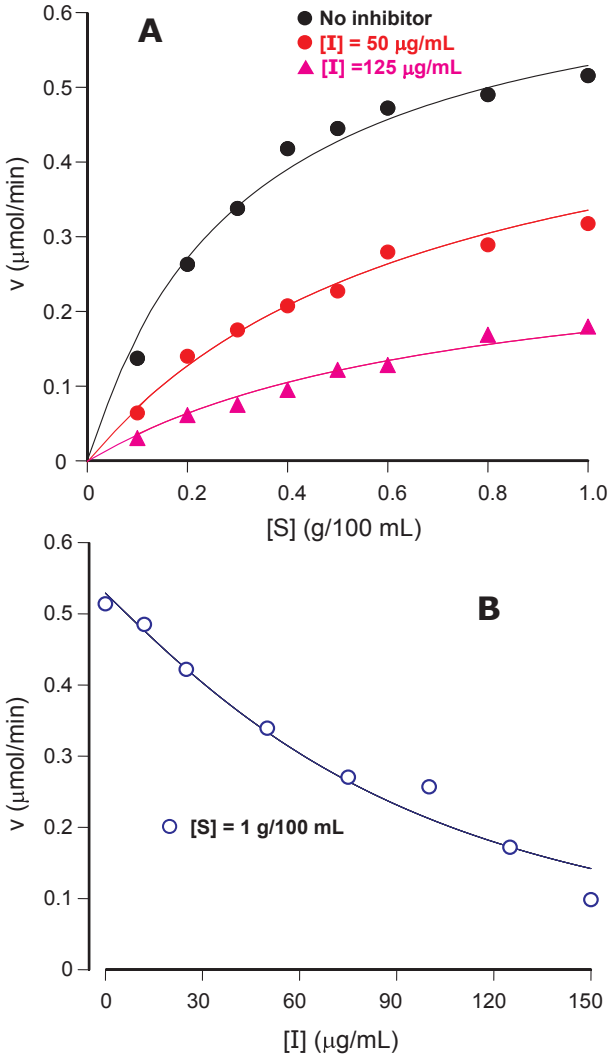

Fig. 2. Reaction rates of the human salivary α-amylase obtained by varying simultaneously the substrate (starch) and the grape pomace extract concentrations. Each datum point is the mean of four determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting Eq. (2) to the experimental data by means of a nonlinear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are K_M, 0.321 ± 0.040 g/100 mL; V_{max}, 0.703 ± 0.032 μmol/min; K_{i1}, 30.75 ± 6.65 μg/mL; K_{i2}, 217.43 ± 129.05 μg/mL; K'_{i1}, 244.71 ± 86.91 μg/mL; sum of squared deviations, 0.0104; MSC, 3.893.

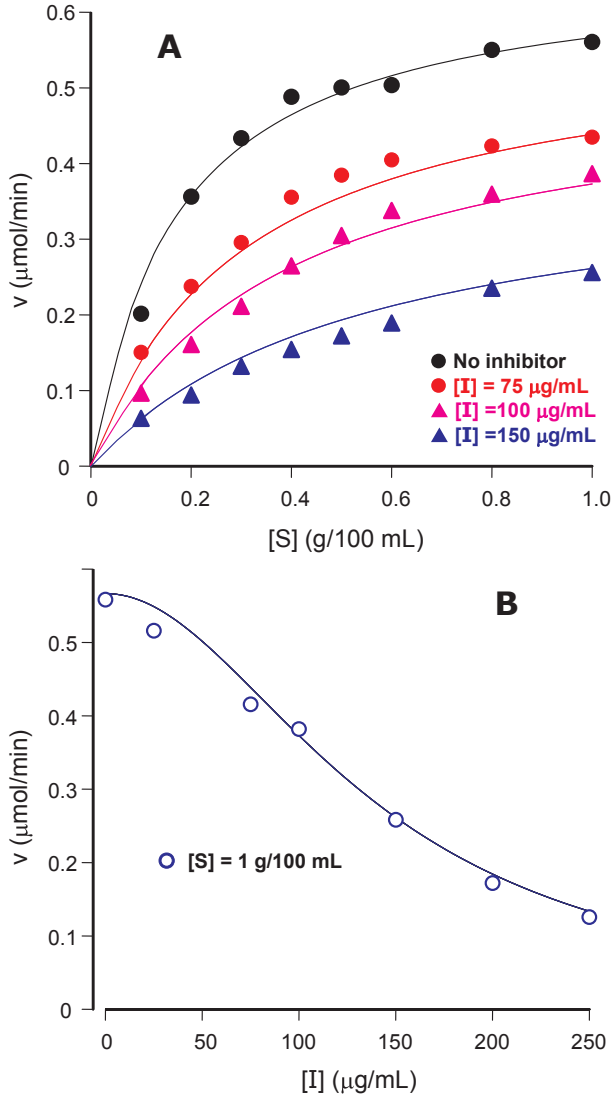

Fig. 3. Reaction rates of the porcine pancreatic α-amylase obtained by varying simultaneously the substrate (starch) and the grape pomace extract concentrations. Each datum point is the mean of four determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting Eq. (3) to the experimental data by means of a nonlinear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are K_M, 0.171 ± 0.017 g/100 mL; V_{max}, 0.663 ± 0.017 μmol/min; $\bar{K}_{i1}$, 72.99 ± 29.00 μg/mL; $\bar{K}_{i2}$, 187.0 ± 84.8.1 μg/mL; sum of squared deviations, 0.0107; MSC, 4.105.

values of K_{i1}, Ki_2 and K'_{i1} indicates that formation of the EI complex is the most likely phenomenon at low concentrations of the inhibitor or mixture of inhibitors as the K_{i1}/K_{i2} ratio is equal to 0.141. Formation of the ESI and EI_2 complexes is less likely, but will certainly occur at concentrations above 100 μg/mL.

The initial rates of the reaction catalyzed by the porcine pancreatic α-amylase at varying substrate and inhibitor concentrations are shown in Fig. 3A. The general pattern is similar to that obtained for the salivary enzyme. Attempts at fitting Eq. (2) to the data failed, however, as failed attempts at fitting several equations containing individualized inhibitor constants. The only equation that could be fitted was one containing the average composite inhibition constants, $\bar{K}_{i1}$ and $\bar{K}_{i2}$, in combination with quadratic [I] terms (Desseaux, Koukielkolo, Moreau, Santimoni, & Marchis-Mouren, 2002; Silva et al., 2014):

$$v = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]^2}{(\bar{K}_{i1})^2}\right) + [S]\left(1 + \frac{[I]^2}{(\bar{K}_{i2})^2}\right)} \quad (3)$$

Indicators of fitting goodness and the optimized values of the various parameters are given in the legend to Fig. 3. Comparison of experiment and theory is allowed by Panels A and B in Fig. 3 because the continuous lines were computed after substituting the optimized parameters into Eq. (3). As in the preceding case of the salivary enzyme, there is a relatively good agreement between theory and experiment, including the v × [I] relationship. Prediction of the inhibition degree at various substrate and inhibitor concentrations by means of Eq. (3) is, thus, a real possibility. The composite inhibition constants $\bar{K}_{i1}$ and $\bar{K}_{i2}$ have a complex meaning (Desseaux et al., 2002; Silva et al., 2014). In principle, they can be taken as indicating that complexes of the type EI_2, ESI_2 or of higher order amply predominate when the MGPE is present in the reaction medium. As it was the case with the salivary

enzyme, complexation with the free pancreatic α-amylase also occurs more easily than binding to the enzyme-substrate complex, as K'_{i1}/K'_{i2} equals 0.39. On the other hand, the values of the inhibitor constants obtained with both enzymes are compatible with the stronger inhibition observed with the salivary α-amylase. Binding of the extract components to the free salivary enzyme, for example, is (72.99/30.75 =) 2.37 times more likely than the same phenomenon with the pancreatic enzyme.

Even considering the heterogeneity of the preparation that was used here, it should be remarked that this fact does not invalidate the use of Eqs. (2) and (3), provided that the proportions between the concentrations of the active compounds are not modified, a condition that holds whenever various quantities of a given preparation are used (Cleland, 1963; Plowman, 1972). The inhibition constants under such circumstances, however, will have a quite complex significance. They are in fact complex functions of several individual dissociation constants and factors relating the total weight of the extract and its content in active principles. Even so they are still a measure of the strength of a given preparation expressed in terms of the extract mass that was added, as can be readily deduced by means of the general algorithms of steady-state enzyme kinetics (Chou & Talalay, 1977; Cleland, 1963; Plowman, 1972). At this point it is worth to compare the results of the present study with those reported by our group about acarbose, a well described inhibitor of α-amylases (Silva et al., 2014). Acarbose is a much stronger inhibitor of both salivary and pancreatic α-amylases, with inhibition constants in the range of up to 10 μg/mL. However, it also binds to both the free and to the enzyme already complexed with the substrate and, in the case of the pancreatic enzyme, the inhibition is parabolic with the formation of complexes EI_2, ESI_2 or higher.

3.2. In silico studies

As mentioned at Introduction, the chemical composition of the MGPE is quite complex (Table 1) and the isolation of the various compounds for testing their inhibitory activity on the α-amylases can be quite laborious. A most convenient approach would be to infer initially from computer simulations what are the most likely compounds (or compound) to be acting as inhibitors of the α-amylases.

As a starting strategy redocking simulations were used for selecting the docking programs and protocols. This procedure consists in docking a ligand that is already present in a crystallographic or modelled structure. If the program succeeds in “understanding” the forces that drive the interactions among protein and ligand, the results will be a best pose that matches the modelled/crystallographic one in all repetitions. A successful redocking simulation suggests that the docking protocol can be used to evaluate unknown ligands from a virtual library. In this work, the redocking method was repeated four times in each program and the results are summarised in Table S1. All three programs were able to redock the acarbose pentasaccharide ligand on the porcine pancreatic α-amylase binding site (Fig. S1). Thus, the docking protocols were applied to evaluate the grape extract molecules present in our virtual library composed by 21 molecules. To select the most promising ligands, we considered not only score values, but also the reproducibility in all repetitions using every program. Since each one uses a distinct set of algorithms to predict binding poses and scores, when different programs can reproduce a similar result repeatedly, it is more likely that they are true-positives. The best results found from virtual screening are displayed in Table 2 and their structures are represented in Fig. S2. A remark should be made with respect to the B-type (epi)catechin dimers, trimers and tetramer in the extract (Table 1). The (epi)catechin pharmacophore group was not selected by virtual screening as a potential candidate for inhibitor. Consequently, there was also no justification for exploring different possibilities for the arrangement of the B-type oligomers.

The molecule that yielded the most promising scores was peonidin-3-*O*-acetylglucoside (P3A, Fig. S3-A), an anthocyanin that belongs to the class of phenolic compounds usually known as flavonoids (Ghosh & Konishi, 2007). Anthocyanins are water-soluble pigments with potent antioxidant properties that provide health benefits in cases of inflammation, obesity and diabetes *mellitus* (Gowd, Jia, & Chen, 2017). There are literature reports that other anthocyanins, such as cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G), could competitively inhibit the porcine pancreatic α-amylase *in vitro*, with IC_{50} values of 24 μM and 75 μM, respectively (Sui, Zhang, & Zhou, 2016). Both P3G and C3G share the same anthocyanin backbone (Fig. S3-B), differing only in the R group on C3′ (B ring), while P3G and P3A differ only in the acetyl group on C6″ (glucoside).

Based on these observations, we decided to further investigate if C3G (which was not identified in the grape Merlot extract), as well as P3G, which is present in the extract, would show similar results as P3A using docking simulations. The structures were evaluated using the validated protocols described earlier and the results of the calculations are displayed in Table S2. In all simulations, P3A showed the highest score and its best pose was similar to C3G and P3G (Fig. S4). The fact that all three anthocyanins assume practically the same poses from all three programs that were used, suggests they are stabilized by the same interactions in the active site, also known as the binding cleft, which lies near the protein centre and can be characterized by the catalytic residues Asp197, Glu233 and Asp300 (Larson, Day, & McPherson, 2010). Fig. 4 shows the P3A docked in the porcine pancreatic α-amylase active site surrounded by side chains residues. All programs showed that the P3A was surrounded by the same three catalytic residues and also in vicinity to the lateral chains of Lys200, Arg195, Gln63 and His305.

Pancreatic α-amylase is a better target for the development of inhibitors with antihyperglycemic activity regarding salivary α-amylase (Qin et al., 2011), given its longer half-life/activity in the duodenum lumen. Furthermore, there is no crystallographic structure of the salivary α-amylase bonded to our control inhibitor ACA deposited in the PDB. Therefore, in our virtual screening simulations, we used pancreatic α-amylase as the main target. However, after virtual screening, the best ranked ligands were equally docked on the salivary α-amylase where they presented poses quite close to that seen in the pancreatic α-amylase, with a characteristic cluster of conformation (Fig. S5).

Based on our docking simulations, it can be deduced that the anthocyanins are able to block the α-amylase catalytic site. In principle, however, this is equally valid for catechin, which is considerably more abundant in the MGPE than any individual anthocyanin (Table 2). Although the affinity of a protein for a ligand can only be accurately estimated by more sensitive calculation methods such as Linear Interaction Energy (LIE) (Gutiérrez-de-Terán & Åqvist, 2012) or Molecular Mechanics Poison-Boltzmann Surface Area (MM-PBSA) (Kumari et al., 2014), the docking simulations provide important clues to find the protein binding site and to understand its interaction with the ligands. Docking simulations also contribute to devise the most likely conformation that a given ligand may assume on binding. The scores obtained for the various ligands described in Table 2 are relatively close. This suggests competition between themselves or with the substrate for the enzyme active site. Although binding is equally possible in the presence of substrate, as indicated by the kinetic data that revealed a mixed type of inhibition, binding in the absence of substrate is considerably stronger, as revealed by the fact that the pertinent apparent dissociation constants for the EI complexes (K_{i1} and $\bar{K}_{i1}$) are smaller for both enzymes than the apparent dissociation constants for the ESI complexes (see legends to Figs. 2 and 3).

The interactions described so far could, thus, explain the inhibitory activity of the Merlot grape pomace extract on the α-amylases and provide useful information for the development of new inhibitors. Even though P3G, C3G and P3A have similar chemical structures and showed overlapping binding poses, P3A seems to have a significantly higher docking score for binding to the active site, based on the results from three different softwares. Catechin, on the other hand, presents

Table 2
Molecules selected from virtual screening after four repetitions using three programs on two α-amylase targets.

Selected compounds	Autodock score	Molegro score	Gold score
Docking using porcine pancreatic α-amylase			
Peonidin-3-*O*-acetylglucoside (P3A)	−8,40 ± 0,14[a]	−156,00 ± 1,90[a]	81,46 ± 0,84[a]
Isorhamnetin-3-*O*-glucoside (I3G)	−7,26 ± 0,27[b]	−131,12 ± 0,20[b]	74,83 ± 1,98[b]
Quercetin-3-*O*-glucuronide (Q3G)	−7,07 ± 0,18[b]	−137,70 ± 0,42[c]	70,47 ± 0,41[c]
Catechin (KTC)	−7,44 ± 0,02[c]	−102,17 ± 0,10[d]	66,12 ± 1,05[d]
Docking using human salivary α-amylase			
Peonidin-3-*O*-acetylglucoside (P3A)	−8.46 ± 0.09[a]	−172.95 ± 4.06[a]	84.89 ± 1.53[a]
Isorhamnetin-3-*O*-glucoside (I3G)	−7.09 ± 0.10[b]	−144.49 ± 0.17[b]	72.27 ± 2.80[b]
Quercetin-3-*O*-glucuronide (Q3G)	−7.38 ± 0.20[c]	−142.11 ± 0.06[c]	70.91 ± 1.94[b]
Catechin (KTC)	−7.17 ± 0.02[b]	−98.88 ± 0.18[d]	63.24 ± 0.41[c]

Different letters in the same column mean statistical difference ($p < 0.05$).

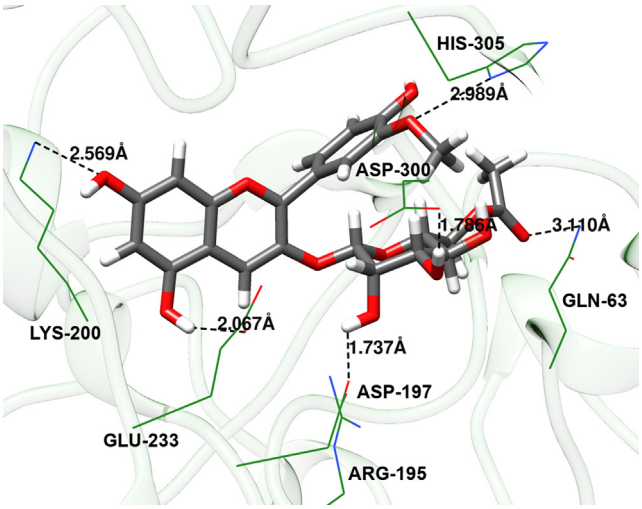

Fig. 4. Best ranked pose of peonidin-3-*O*-acetylglucoside (P3A) docked on the catalytic binding site of the porcine pancreatic α-amylase. The catalytic residues Asp197, Asp300 and Glu233 are in close contact with P3A.

relatively lower docking scores compared to the three anthocyanins (Table 2), but its relative abundance in the MGPE (Table 1) does not allow to exclude its participation in the inhibitory activity.

3.3. In vivo inhibition of the activities of carbohydrate digestive enzymes by the grape pomace extract

It is important to find out if the effects of MGPE on the α-amylases have physiological relevance, i.e., if they also occur *in vivo*. An experimental approach that is likely to produce an answer to this question is to quantify the appearance of glucose in blood after feeding animals with starch. Similarly, due to the virtual absence of effects on α-glucosidase, no modifications in blood glucose levels should occur upon maltose administration. This reasoning resides on the generally accepted notion that the action of hydrolytic enzymes in the intestinal tract is essential for the absorption of the individual molecular constituents of di-, oligo- and polysaccharides. Table 1 shows the daily doses of phenolic non-anthocyanins and anthocyanins compounds that were automatically administered as part of the 50 or 250 mg extract per kg weight that were given to the animals.

Figs. 5–7 illustrate the results of the experiments that were done in the search for answers regarding the question formulated above. Administration of starch alone (Fig. 5A) produced the control curve in which the blood glucose concentration increased up to a maximal value (at 30 min) which was 90% above the basal concentration. The declining tendency was yet not complete at 60 min as the glucose concentration was still considerably above the basal line. Water administration did not perturb the blood glucose concentrations in a significant way during the period of 60 min. A smaller increase in the blood levels of glucose was found when starch and 50 mg/kg of MGPE were given simultaneously ($p \leq 0.05$). When the MGPE dose was raised to 250 mg/kg, the events were somewhat modified with an initial delay and smaller levels at the end (60 min). The effects of this dose were close to the modifications caused by acarbose. Panel B in Fig. 4 shows the areas under the various time-response curves obtained after starch administration under various conditions, diminished from area under the curve when solely water was administered. Both MGPE doses significantly reduced the areas under the curves. The diminutions at the doses of 50 and 240 mg/kg performed, respectively, 37.1 and 71.4%.

Contrarily to what was observed with starch, simultaneous administration of MGPE and maltose did not cause any reduction in blood glucose levels (Fig. 6). This observation is consistent with the *in vitro* assays, in which the α-glucosidase activity was not negatively affected by the extract (Fig. 1). Finally, Fig. 7 shows that MGPE had no inhibitory effect when administered simultaneously with glucose. These *in vivo* studies reinforce the idea that the grape pomace extract acts by inhibiting amylase activity, but does not present any inhibitory action on alpha-glucosidase and does also not interfere with glucose absorption.

The phenolic composition of the grape pomace extract used in this work has already been described by Corrêa et al. (2017) and Gonçalves et al. (2017). The most abundant phenolic compounds were epicatechin, catechin, quercetin, myricetin, isorhamnetin glycoside derivatives, malvidin-3-*O*-glucoside and peonidin-3-*O*-glucoside. The probable participation of catechin and several anthocyanins in the inhibition of α-amylases has already been discussed above. Considering the type of molecules found in the extract, the results obtained in this study partially diverge with the general concept that phenolic compounds, such as flavonoids and polyphenols, have the ability of inhibiting the α-glucosidase activity (Ishikawa et al., 2007; Hargrove, Greenspan, Hartle, & Dowd, 2011; Kumar, Narwal, Kumar, & Prakash, 2011; Yin, Zhang, Feng, Zhang, & Kang, 2014; Kadouh, Sun, Zhu, & Zhou, 2016; Martinez-Gonzalez et al., 2017; Li, Wang, et al., 2019; Zhu et al., 2020). It should be stressed, however, that there are reports showing that the intestinal enzyme and the yeast enzyme generally present different sensitivities to flavonoids and polyphenols (Babu et al., 2004; Tadera, Minami, Takamatsu, & Matsuoka, 2006). Tadera et al. (2006), for example, reports that the rat intestinal α-glucosidase is slightly inhibited by a great number of flavonoids, and only minimally by anthocyanidin and isoflavone groups. The yeast enzyme, on the contrary, is strongly inhibited by isoflavone, anthocyanidin and flavonol groups. It seems, thus, that the grape pomace extract used in the present work presents a combination of constituents that are inactive on the intestinal α-glucosidase within the concentration range that was used. Furthermore, there are reports that the substrate may equally influence the degree of inhibition (Tadera et al., 2006). In the present study, maltose was used because this is the physiological substrate. In many studies in the past, however, synthetic substrates such as *p*-nitrophenyl-α-D-

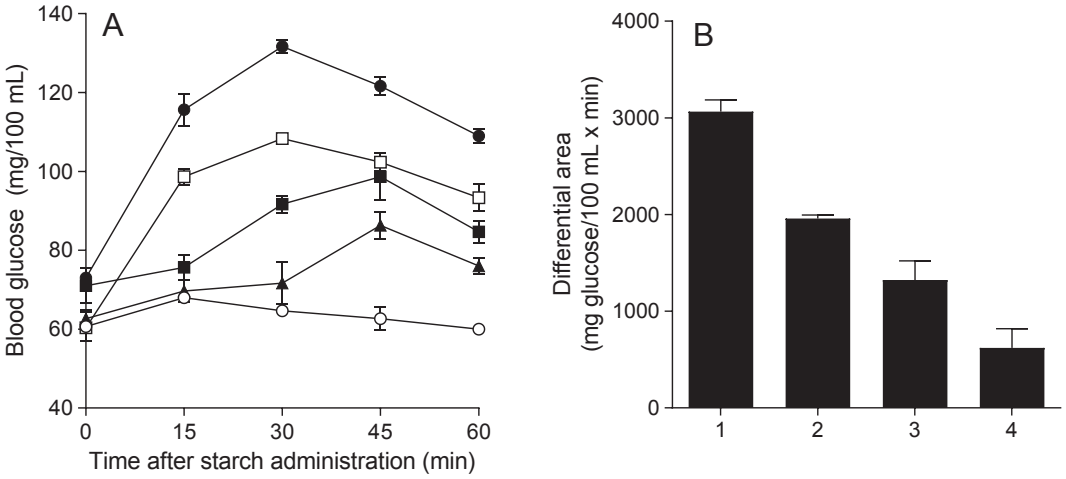

Fig. 5. Influence of MGPE and acarbose administration on the glycemic levels of fasted rats during 60 min following starch administration. Blood samples from the tail vein were analyzed by means of a glucometer after intragastric starch administration (1 g per kg body weight). Each datum point represents the mean ± mean standard errors of three experiments. (●,1) Starch alone; (○) water alone; (▲, 4) starch plus 50 mg/kg acarbose; (□,2) starch plus 50 mg/kg MGPE; (■,3) starch plus 250 mg/kg MGPE. Experimental details are given in the Materials and methods section. In B, all values are statistically different from each other ($p \leq 0.05$).

glucopyranoside were used a fact that might have influenced the inhibition degree.

The results of the present study add both *in vitro* and *in vivo* evidence about the action of flavonoids and other polyphenolics of various kinds on postprandial glycemia after the ingestion of starch, but not maltose or glucose (Silva et al., 2014; Kato et al., 2017; Martinez-Gonzalez et al., 2017; Kato-Schwartz et al., 2018; Shanmugam et al., 2018; Gutiérrez-Grijalva et al., 2019; Li, Wang, et al., 2019; Cardullo et al., 2020; Zhu et al., 2020). That flavonoids and polyphenolics in general may affect glycemia by inhibiting starch hydrolyzing enzymes *in vivo* is a widespread notion (Sun et al., 2019). An alternative or complementary mechanism that has been suggested involves inhibition of glucose transfer from the intestinal lumen to the cells, which is accomplished by glucose transporters in the enterocytes of the small intestine (Sun et al., 2019). A direct inhibitory action on these transporters was excluded by the glucose tolerance experiments in the present work, which revealed no diminutions in the rate of glucose appearance in blood after the MGPE administration. It still remains the possibility of an inhibition of the expression of the glucose transporters (Sun et al., 2019). In the present work, however, only short-term effects were examined and gene expressions are medium- or long-term phenomena. Clearly, this is a question that has to be answered by future specific experiments.

4. Conclusion

In conclusion, MGPE can be regarded as an inhibitor of both the salivary and the pancreatic α-amylases, but not of the α-glucosidase enzyme. Kinetically the inhibition showed a complex pattern, with multiple binding of the extract constituents to the enzymes. Furthermore, the *in silico* docking studies indicated that some anthocyanin compounds, such as peonidin-3-*O*-acetylglucoside, quercetin-3-*O*-glucuronide and isorhamnetin-3-*O*-glucoside, in addition to catechin, were the most likely polyphenols responsible for the α-amylase inhibition caused by MGPE. Finally, MGPE had also a negative effect on starch hydrolysis *in vivo*, as it diminished the increases in glucose levels that followed starch administration. These findings are supportive to the hypothesis that MGPE could adequate as an adjuvant for keeping normal glycemia and may serve as the basis for clinical trials.

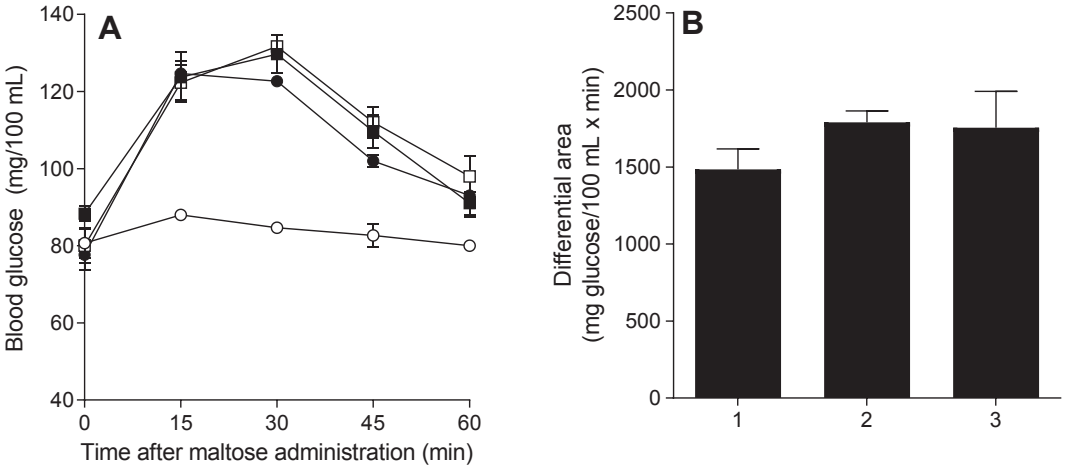

Fig. 6. Influence of MGPE administration on the glycemic levels of fasted rats during 60 min following maltose administration. Blood samples from the tail vein were analyzed by means of a glucometer after intragastric starch administration (1 g per kg body weight). Each datum point represents the mean ± mean standard errors of three experiments. (●,1) Maltose alone; (○) water alone; (□,2) maltose plus 250 mg/kg MGPE; (■,3) maltose plus 500 mg/kg MGPE. Experimental details are given in the Materials and methods section. In B, all values are not statistically different from each other ($p \leq 0.05$).

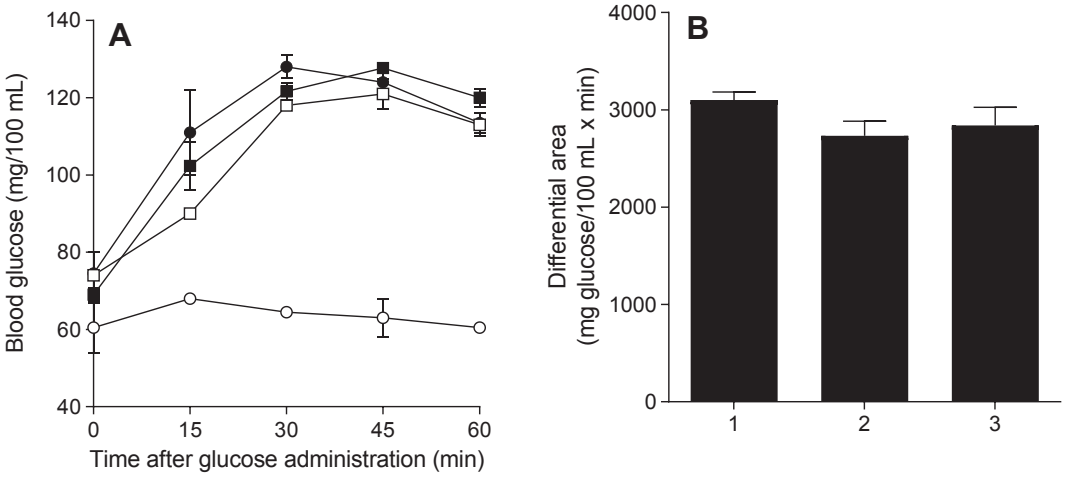

Fig. 7. Influence of MGPE administration on the glycemic levels of fasted rats during 60 min following glucose administration. Blood samples from the tail vein were analyzed by means of a glucometer after intragastric glucose administration (1 g per kg body weight). Each datum point represents the mean ± mean standard errors of three experiments. (●,1) Glucose alone; (○) water alone; (■) glucose plus 100 mg/kg MGPE; (■,2) glucose plus 500 mg/kg MGPE (□,3). Experimental details are given in the Materials and methods section. In B, all values are not statistically different from each other ($p \leq 0.05$).

CRediT authorship contribution statement

Camila Gabriel Kato-Schwartz: Methodology, Investigation. **Rúbia Carvalho Gomes Corrêa:** Methodology, Investigation. **Diego de Souza Lima:** Methodology, Investigation. **Anacharis Babeto de Sá-Nakanishi:** Investigation, Supervision. **Geferson de Almeida Gonçalves:** Methodology, Investigation. **Flavio Augusto Vicente Seixas:** Conceptualization, Supervision. **Charles W.I. Haminiuk:** Conceptualization, Visualization. **Lillian Barros:** Conceptualization, Visualization. **Isabel C.F.R. Ferreira:** Conceptualization, Visualization. **Adelar Bracht:** Writing - review & editing. **Rosane Marina Peralta:** Conceptualization, Writing - review & editing.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109462.

References

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control, 19*, 716–723. https://doi.org/10.1109/TAC.1974.1100705.

Babu, K. S., Tiwari, A. K., Srinivas, P. V., Ali, A. Z., Raju, B. C., & Rao, J. M. (2004). Yeast and mammalian α-glucosidase inhibitory constituents from Himalayan rhubarb *Rheum emodi* Wall.ex Meisson. *Bioorganic & Medicinal Chemistry Letters, 14*, 3841–3845. https://doi.org/10.1016/j.bmcl.2004.04.062.

Balisteiro, D. M., de Araujo, R. L., Giacaglia, L. R., & Genovese, M. I. (2017). Effect of clarified Brazilian native fruit juices on postprandial glycemia in healthy subjects. *Food Research International, 100*, 196–203. https://doi.org/10.1016/j.foodres.2017.08.044.

Barba, F. J., Zhu, Z., Koubaa, M., Sant'Ana, A. S., & Orlien, V. (2016). Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review. *Trends in Food Science & Technology, 49*, 96–109. https://doi.org/10.1016/j.tifs.2016.01.006.

Beres, C., Costa, G. N., Cabezudo, I., da Silva-James, N. K., Teles, A. S., Cruz, A. P., ... Freitas, S. P. (2017). Towards integral utilization of grape pomace from winemaking process: A review. *Waste management, 68*, 581–594. https://doi.org/10.1016/j.wasman.2017.07.017.

Campbell, I. (2007). Oral antidiabetic drugs: Their properties and recommended use. *Prescriber, 18*, 56–74. https://doi.org/10.1002/psb.48.

Cardullo, N., Muccilli, V., Pulvirenti, L., Cornu, A., Pouységu, L., Deffieux, D., ... Tringali, C. (2020). C-glucosidic ellagitannins and galloylated glucoses as potential functional food ingredients with anti-diabetic properties: A study of α-glucosidase and α-amylase inhibition. *Food Chemistry, 313*, Article 126099. https://doi.org/10.1016/j.foodchem.2019.126099.

Chou, T. C., & Talalay, P. (1977). A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. *Journal of Biological Chemistry, 252*, 6438–6442.

Cleland, W. W. (1963). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: Nomenclature and theory. *Biochimica and Biophysical Acta, 67*, 173–187. https://doi.org/10.1016/0926-6569(63)90226-8.

Corrêa, R. C. G., Haminiuk, C. W., Barros, L., Dias, M. I., Calhelha, R. C., Kato, C. G., ... Ferreira, I. C. (2017). Stability and biological activity of Merlot (*Vitis vinifera*) grape pomace phytochemicals after simulated in vitro gastrointestinal digestion and colonic fermentation. *Journal of Functional Foods, 36*, 410–417. https://doi.org/10.1016/j.jff.2017.07.030.

Corrêa, R. C. G., Peralta, R. M., Haminiuk, C. W., Maciel, G. M., Bracht, A., & Ferreira, I. C. (2018). New phytochemicals as potential human anti-aging compounds: Reality, promise, and challenges. *Critical reviews in food science and nutrition, 58*, 942–957. https://doi.org/10.1080/10408398.2016.1233860.

Dallakyan, S., & Olson, A. J. (2015). Small-molecule library screening by docking with PyRx. *Chemical biology* (pp. 243–250). New York, NY: Humana Press. https://doi.org/10.1007/978-1-4939-2269-7_19.

Desseaux, V., Koukielkolo, R., Moreau, Y., Santimoni, M., & Marchis-Mouren, G. (2002). Mechanism of porcine pancreatic α-amylase: Inhibition of amylose and maltopentaose hydrolysis by various inhibitors. *Biologia, Bratislava, 57*, 163–170.

Drevelegka, I., & Goula, A. M. (2020). Recovery of grape pomace phenolic compounds through optimized extraction and adsorption processes. *Chemical Engineering and Processing-Process Intensification, 107845*. https://doi.org/10.1016/j.cep.2020.107845.

Dulf, F. V., Vodnar, D. C., Toşa, M. I., & Dulf, E. H. (2020). Simultaneous enrichment of grape pomace with γ-linolenic acid and carotenoids by solid-state fermentation with Zygomycetes fungi and antioxidant potential of the bioprocessed substrates. *Food Chemistry, 310*, Article 125927. https://doi.org/10.1016/j.foodchem.2019.125927.

Emsley, P., Lohkamp, B., Scott, W. G., & Cowtan, K. (2010). Features and development of Coot. *Acta Crystallographica Section D: Biological Crystallography, 66*, 486–501. https://doi.org/10.1107/S0907444910007493.

Giovannini, P., Howes, M. J. R., & Edwards, S. E. (2016). Medicinal plants used in the traditional management of diabetes and its sequelae in Central America: A review. *Journal of Ethnopharmacology, 184*, 58–71. https://doi.org/10.1016/j.jep.2016.02.034.

Ghosh, D., & Konishi, T. (2007). Anthocyanins and anthocyanin-rich extracts: Role in diabetes and eye function. *Asia Pacific Journal of Clinical Nutrition, 16*, 200–208.

Gonçalves, G. A., Soares, A. A., Correa, R. C., Barros, L., Haminiuk, C. W., Peralta, R. M., ... Bracht, A. (2017). Merlot grape pomace hydroalcoholic extract improves the oxidative and inflammatory states of rats with adjuvant-induced arthritis. *Journal of Functional Foods, 33*, 408–418. https://doi.org/10.1016/j.jff.2017.04.009.

Gowd, V., Jia, Z., & Chen, W. (2017). Anthocyanins as promising molecules and dietary bioactive components against diabetes–A review of recent advances. *Trends in Food*

Science & Technology, 68, 1–13. https://doi.org/10.1016/j.tifs.2017.07.015.
Gutiérrez-de-Terán, H., & Åqvist, J. (2012). Linear interaction energy: Method and applications in drug design. *Computational Drug Discovery and Design* (pp. 305–323). New York, NY: Springer. https://doi.org/10.1007/978-1-61779-465-0_20.
Gutiérrez-Grijalva, E. P., Antunes-Ricardo, M., Acosta-Estrada, B. A., Gutiérrez-Uribe, J. A., & Heredia, J. B. (2019). Cellular antioxidant activity and in vitro inhibition of α-glucosidase, α-amylase and pancreatic lipase of oregano polyphenols under simulated gastrointestinal digestion. *Food Research International, 116*, 676–686. https://doi.org/10.1016/j.foodres.2018.08.096.
Hamden, K., Jaouadi, B., Carreau, S., Aouidet, A., & Elfeki, A. (2011). Therapeutic effects of soy isoflavones on α-amylase activity, insulin deficiency, liver–kidney function and metabolic disorders in diabetic rats. *Natural Product Research, 25*, 244–255. https://doi.org/10.1080/14786411003683117.
Hargrove, J. L., Greenspan, P., Hartle, D. K., & Dowd, C. (2011). Inhibition of aromatase and α-amylase by flavonoids and proanthocyanidins from sorghum bicolor bran extracts. *Journal of Medicinal Food, 14*, 799–807. https://doi.org/10.1089/jmf.2010.0143.
Ishikawa, A., Yamashita, H., Hiemori, M., Inagaki, E., Kimoto, M., Okamoto, M., ... Natori, Y. (2007). Characterization of inhibitors of postprandial hyperglycemia from the leaves of *Nerium indicum*. *Journal of Nutritional Science and Vitaminology, 53*, 166–173. https://doi.org/10.3177/jnsv.53.166.
Jara-Palacios, M. J., Hernanz, D., Cifuentes-Gomez, T., Escudero-Gilete, M. L., Heredia, F. J., & Spencer, J. P. (2015). Assessment of white grape pomace from winemaking as source of bioactive compounds, and its antiproliferative activity. *Food Chemistry, 183*, 78–82. https://doi.org/10.1016/j.foodchem.2015.03.022.
Jones, G., Willett, P., Glen, R. C., Leach, A. R., & Taylor, R. (1997). Development and validation of a genetic algorithm for flexible docking. *Journal of Molecular Biology, 267*, 727–748. https://doi.org/10.1006/jmbi.1996.0897.
Kadouh, H. C., Sun, S., Zhu, W., & Zhou, K. (2016). α-Glucosidase inhibiting activity and bioactive compounds of six red wine grape pomace extracts. *Journal of Functional Foods, 26*, 577–584. https://doi.org/10.1016/j.jff.2016.08.022.
Kato, C. G., Gonçalves, G. D. A., Peralta, R. A., Seixas, F. A. V., de Sá-Nakanishi, A. B., Bracht, L., ... Peralta, R. M. (2017). Inhibition of α-amylases by condensed and hydrolysable tannins: Focus on kinetics and hypoglycemic actions. *Enzyme Research, ID 5724902*. https://doi.org/10.1155/2017/5724902.
Kato-Schwartz, C. G., Bracht, F., de Almeida Gonçalves, G., Soares, A. A., Vieira, T. F., Brugnari, T., ... Peralta, R. M. (2018). Inhibition of α-amylases by pentagalloyl glucose: Kinetics, molecular dynamics and consequences for starch absorption. *Journal of Functional Foods, 44*, 265–273. https://doi.org/10.1016/j.jff.2018.03.025.
Kato-Schwartz, C. G., Sá-Nakanishi, A. B., Guidi, A. C., Gonçalves, G. A., Bueno, F. G., Zani, B. P. M., ... Peralta, R. M. (2020). Carbohydrate digestive enzymes are inhibited by *Poincianella pluviosa* stem bark extract: Relevance on type 2 diabetes treatment. *Clinical Phytoscience, 6*, 31. https://doi.org/10.1186/s40816-020-00177-w.
Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B. A., Thiessen, P. A., Yu, B., Zaslavsky, L., Zhang, J., & Bolton, E. E. (2019). PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Research, 47*, D1102–D1109. https://doi.org/10.1093/nar/gky1033.
Kumar, S., Narwal, S., Kumar, V., & Prakash, O. (2011). α-glucosidase inhibitors from plants: A natural approach to treat diabetes. *Pharmacognosy Reviews, 5*, 19–29. https://doi.org/10.4103/0973-7847.79096.
Kumari, R., Kumar, R., Open Source Drug Discovery Consortium, & Lynn, A. (2014). g_mmpbsa - A GROMACS tool for high-throughput MM-PBSA calculations. *Journal of Chemical Information and Modeling, 54*, 1951–1962. https://doi.org/10.1021/ci500020m.
Lalegani, S., Gavlighi, H. A., Azizi, M. H., & Sarteshnizi, R. A. (2018). Inhibitory activity of phenolic-rich pistachio green hull extract-enriched pasta on key type 2 diabetes relevant enzymes and glycemic index. *Food Research International, 105*, 94–101. https://doi.org/10.1016/j.foodres.2017.11.003.
Larson, S. B., Day, J. S., & McPherson, A. (2010). X-ray crystallographic analyses of pig pancreatic α-amylase with limit dextrin, oligosaccharide, and α-cyclodextrin. *Biochemistry, 49*, 3101–3115. https://doi.org/10.1021/bi902183w.
Li, C., Begum, A., Numao, S., Park, K. H., Withers, S. G., & Brayer, G. D. (2005). Acarbose rearrangement mechanism implied by the kinetic and structural analysis of human pancreatic α-amylase in complex with analogues and their elongated counterparts. *Biochemistry, 44*, 3347–3357. https://doi.org/10.1021/bi048334e.
Li, R. Y., Wang, S., McClements, D. J., Wan, Y., Liu, C. M., & Fu, G. M. (2019). Antioxidant activity and α-amylase and α-glucosidase inhibitory activity of a fermented tannic acid product: Trigalloylglucose. *LWT Food Science and Technology, 112*, Article 108249. https://doi.org/10.1016/j.lwt.2019.108249.
Li, D., Sun, L., Yang, Y., Wang, Z., Yang, X., Zhao, T., ... Guo, Y. (2019). Young apple polyphenols postpone starch digestion in vitro and in vivo. *Journal of Functional Foods, 56*, 127–135. https://doi.org/10.1016/j.jff.2019.03.009.
Liu, M., Hu, B., Zhang, H., Zhang, Y., Wang, L., Qian, H., & Qi, X. (2017). Inhibition study of red rice polyphenols on pancreatic α-amylase activity by kinetic analysis and molecular docking. *Journal of Cereal Science, 76*, 186–192. https://doi.org/10.1016/j.jcs.2017.04.011.
Ma, Q., Li, Y., Li, P., Wang, M., Wang, J., Tang, Z., ... Zhao, B. (2019). Research progress in the relationship between type 2 diabetes mellitus and intestinal flora. *Biomedicine & Pharmacotherapy, 117*, Article 109138. https://doi.org/10.1016/j.biopha.2019.109138.
Martinez-Gonzalez, A., Diaz-Sánchez, A. G., de la Rosa, L. A., Vargas-Requena, C. L., Bustos-Jaimes, I., & Alvarez-Parrilla, E. (2017). Polyphenolic compounds and digestive enzymes: *In vitro* non-covalent interactions. *Molecules, 22*, 669–693. https://doi.org/10.3390/molecules22040669.
Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426–428. https://doi.org/10.1021/ac60147a030.
Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry, 30*, 2785–2791. https://doi.org/10.1002/jcc.21256.
Mumtaz, M. W., Al-Zuaidy, M. H., Abdul Hamid, A., Danish, M., Akhtar, M. T., & Mukhtar, H. (2018). Metabolite profiling and inhibitory properties of leaf extracts of Ficus benjamina towards α-glucosidase and α-amylase. *International Journal of Food Properties, 21*, 1560–1574. https://doi.org/10.1080/10942912.2018.1499112.
Peixoto, C. M., Dias, M. I., Alves, M. J., Calhelha, R. C., Barros, L., Pinho, S. P., & Ferreira, I. C. (2018). Grape pomace as a source of phenolic compounds and diverse bioactive properties. *Food Chemistry, 253*, 132–138. https://doi.org/10.1016/j.foodchem.2018.01.163.
Plowman, K. M. (1972). *Enzyme kinetics.* New York: McGraw-Hill Book Company.
Qin, X., Ren, L., Yang, X., Bai, F., Wang, L., Geng, P., ... Shen, Y. (2011). Structures of human pancreatic α-amylase in complex with acarviostatins: Implications for drug design against type II diabetes. *Journal of Structural Biology, 174*, 196–202. https://doi.org/10.1016/j.jsb.2010.11.020.
Ribeiro, L. F., Ribani, R. H., Francisco, T. M. G., Soares, A. A., Pontarolo, R., & Haminiuk, C. W. I. (2015). Profile of bioactive compounds from grape pomace *(Vitis vinifera* and *Vitis labrusca*) by spectrophotometric, chromatographic and spectral analyses. *Journal of Chromatography B, 1007*, 72–80. https://doi.org/10.1016/j.jchromb.2015.11.005.
Sales, P. M., Souza, P. M., Simeoni, L. B., Magalhães, P. O., & Dâmaris, S. (2012). α-Amylase inhibitors: A review of raw material and isolated compounds from plant source. *Journal of Pharmaceutical Sciences, 15*, 141–183. https://doi.org/10.18433/j35s3k.
Silva, S. M., Koehnlein, E. A., Bracht, A., Castoldi, R., de Morais, G. R., Baesso, M. L., ... Peralta, R. M. (2014). Inhibition of salivary and pancreatic α-amylases by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. *Food Research International, 56*, 1–8. https://doi.org/10.1016/j.foodres.2013.12.004.
Shanmugam, S., Gomes, I. A., Denadai, M., dos Santos Lima, B., de Souza Araújo, A. A., Narain, N., ... Thangaraj, P. (2018). UHPLC-QqQ-MS/MS identification, quantification of polyphenols from *Passiflora subpeltata* fruit pulp and determination of nutritional, antioxidant, α-amylase and α-glucosidase key enzymes inhibition properties. *Food Research International, 108*, 611–620. https://doi.org/10.1016/j.foodres.2018.04.006.
Sui, X., Zhang, Y., & Zhou, W. (2016). In vitro and in silico studies of the inhibition activity of anthocyanins against porcine pancreatic α-amylase. *Journal of Functional Foods, 21*, 50–57. https://doi.org/10.1016/j.jff.2015.11.042.
Sun, L., Warren, F. J., & Gidley, M. J. (2019). Natural products for glycaemic control: Polyphenols as inhibitors of alpha-amylase. *Trends in Food Science & Technology, 91*, 262–273. https://doi.org/10.1016/j.tifs.2019.07.009.
Sterling, T., & Irwin, J. J. (2015). ZINC 15–ligand discovery for everyone. *Journal Of Chemical Information and Modeling, 55*, 2324–2337. https://doi.org/10.1021/acs.jcim.5b00559.
Tadera, K., Minami, Y., Takamatsu, K., & Matsuoka, T. (2006). Inhibition of α-glucosidase and α-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology, 52*, 149–153. https://doi.org/10.3177/jnsv.52.149.
Thomsen, R., & Christensen, M. H. (2006). MolDock: A new technique for high-accuracy molecular docking. *Journal of Medicinal Chemistry, 49*, 3315–3321. https://doi.org/10.1021/jm051197e.
Tresserra-Rimbau, A., Lamuela-Raventos, R. M., & Moreno, J. J. (2018). Polyphenols, food and pharma. Current knowledge and directions for future research. *Biochemical Pharmacology, 156*, 186–195. https://doi.org/10.1016/j.bcp.2018.07.050.
Yin, Z., Zhang, W., Feng, F., Zhang, Y., & Kang, W. (2014). α-Glucosidase inhibitors isolated from medicinal plants. *Food Science and Human Wellness, 3*, 136–174. https://doi.org/10.1016/j.fshw.2014.11.003.
Zhao, X., Zhang, S. S., Zhang, X. K., He, F., & Duan, C. Q. (2020). An effective method for the semi-preparative isolation of high-purity anthocyanin monomers from grape pomace. *Food Chemistry, 310*, Article 125830. https://doi.org/10.1016/j.foodchem.2019.125830.
Zhu, J., Chen, C., Zhang, B., & Huang, Q. (2020). The inhibitory effects of flavonoids on α-amylase and α-glucosidase. *Critical Reviews in Food Science and Nutrition, 60*, 695–708. https://doi.org/10.1080/10408398.2018.1548428.