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ORIGINAL ARTICLE

Anxiolytic and Hypoglycemic Effect of Pectins from Galician Lemon Citrus Fruit on Adult Zebrafish (*Danio rerio*): An *In Vivo* and *In Sílico* Approach

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Abstract
Diabetes mellitus is a medical condition characterized by elevated blood glucose levels, known as hyperglycemia, which can damage other organs of the human body through metabolic alterations. Pectin, a naturally occurring polysaccharide, is present in the cell walls of plants and is composed of a polymer chain of galacturonic acid with additional sugar branches. Pectins, which are more often found in citrus fruits, have been gaining prominence in the pharmacological sector and are widely applied in the food industry. Thus, the main objectives of this study were to obtain amidated pectin from Galician lemon, evaluate its hypoglycemic and anxiolytic potential, and perform a molecular docking study. Pectin from Galician lemon was extracted with ammonium oxalate, and a glucose soak test was performed on zebrafish to determine the potential of pectin from Galician lemon in the treatment of hyperglycemia. A computational study was performed using the AutoDock Vina software and employing the molecular docking technique to prove the efficiency of pectin from Galician lemon as a hypoglycemic agent in comparison with α-acarbose (a reference inhibitor) and metformin (a reference drug). The zebrafish assay showed that pectin had a similar effect to metformin (positive control), reducing the animals' baseline blood glucose levels. The results of the molecular docking study proved that pectin had a higher affinity than α-acarbose and metformin. In addition, pectin demonstrated anxiolytic effects via GABAergic neurotransmission, like the positive control, diazepam.

Keywords *Citrus aurantiifolia* · Pectin · Zebrafish · Natural Products

Introduction

Diabetes mellitus is a dysfunctional glucose metabolic disease characterized by hyperglycemia and hypoglycemia. These metabolic alterations can cause damage to the organs of the human body (Kodl and Seaquist 2008). A method for evaluating the effectiveness of hypoglycemic drugs has been developed for rodents and has also been used in zebrafish, which has substantial potential as a model organism for investigating hyperglycemia and its complications (Shin 2012; Capiotti et al. 2014). Similar to rodents, a hyperglycemic state can be induced in zebrafish through the destruction of pancreatic beta cells (Shin 2012).

Another protocol involves immersing zebrafish in a glucose solution based on the assumption that fish can easily absorb water molecules and regulate their internal water concentrations and total solutes (Gleeson et al. 2007; Capiotti et al. 2014; Dos Santos et al. 2018). According to Shin et al. (2012), the absorption/uptake of glucose occurs through a glucose transporter called GLUT, expressed in the gills (GLUT 1–3, 6, 8, and 10–13) and in the intestine (GLUT 5 and 9) of the zebrafish.

A study carried out by Capiotti et al. (2014) with zebrafish to assess the effects of hyperglycemia on memory and

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acetylcholinesterase activity used the glucose immersion method to induce hyperglycemia in zebrafish. As their genomic structure is similar to that of humans, zebrafish models have been used to investigate a wide range of human pathologies, including genetic and acquired diseases (Ferreira et al. 2021).

Pectin is a complex and amorphous polysaccharide composed of galacturonic acid, with sugars linked by side chains. However, the source and the techniques used in isolation and purification strongly affect its chemical structure, resulting in esterification, methoxylation, or amidation (Chandrarathna et al. 2020). As a natural, biodegradable, biocompatible, and nontoxic polymer, pectin is widely used in the food and cosmetics industries as a thickener, gelling agent, and stabilizer (Noreen et al. 2017). Owing to its bioactivities, it has garnered attention within the pharmaceutical field, showing potential as an anticancer agent (Wang et al. 2014), exhibiting hypoglycemic (Zhan et al. 2019) and hypocholesterolemic activities, and showing promise in the development of controlled-release drug delivery systems (Gadalla et al. 2016).

Therefore, considering the potential of pectin and the obtained results, this study aimed to carry out a test to determine its activity against anxiety and hypoglycemia (through immersion in glucose) in zebrafish. This investigation was carried out using amidated citrus fruit pectin and involved analyzing the direct interactions between the enzyme and hypoglycemia to find the active site of the receptor and validate the results regarding hyperglycemia obtained by a molecular docking analysis.

Materials and Methods

Drugs and Reagents

The following substances were used: glucose (Sigma Chemical Corp.), metformin (Neo Química®), and dimethylsulfoxide (DMSO; Dynamic®).

Identification of Plant Material

Galician lemons were purchased from supermarkets in Fortaleza, CE. Botanical identification of fresh fruits was performed by Dr. Eliseu Marlônio de Lucena, and its voucher specimen was registered in the Herbarium Prisco Bezerra of the Federal University of Ceará, under registration number 52536. The fruits were washed, and the skin was separated manually. Subsequently, the fruits were subjected to freeze-drying and extraction.

Extraction and Characterization of Amidated Pectin

For the extraction of lemon pectin (PECLG), the methodology described by Koubala et al. (2008) was followed, with adaptations. Pectin extraction was conducted using a 0.25% ammonium oxalate solution with a pH of 4.6. The ratio of solution to peels was 5:1, with 40 g of peels used for 200 ml of solvent. The extraction process was carried out at a temperature of 80 °C for a duration of 1 h. First, the obtained extract was filtered, and the process described above was repeated four times. Finally, the filtered extracts were combined, and the pH was adjusted to 6 using a 0.1 M sodium hydroxide solution. The resulting mixture was then concentrated in a vacuum-rotary evaporator. For pectin precipitation, 100 ml of 95% ethanol was added to the concentrate. The precipitate was frozen and lyophilized to obtain pectin, and the yield was calculated.

Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared Absorption Spectroscopy (FT-IR)

Nuclear magnetic resonance spectra were acquired on an Agilent DD2 600 MHz spectrometer equipped with One Probe and a 5 mm internal diameter probe (H-F/15N-31P). The sample was prepared by dissolving 10 mg in 600 µl of deuterated water (D_2O) containing 1% sodium 2,2,3,3-d_4-(3-trimethylsilyl)-propionate (TMSP-*d4*) as internal reference. The unidimensional experiments ^{1}H and ^{13}C NMR were performed at 60 °C using standard pulse sequences. Additionally, chemical shifts (δ) were expressed in ppm, adapted from Synytsya et al. (2003).

Determination of the Degree of Esterification by FT-IR

In the infrared spectrum of amidated pectin, the area under the bands corresponding to the esterified carboxylic groups —COO-R (1760–1745 cm^{-1}) and the area under the band corresponding to the free carboxylic groups COO— (1640–1620 cm^{-1}) were used to calculate the degree of esterification (DE) of the sample. Equation 1 was used to calculate the DE of the pectin sample using FTIR. The integration areas were calculated using Origin 8 software (Šimkovic et al. 2009).

$$\%DE = \frac{esterified\ groups}{esterified\ groups + carboxylate\ groups} \times 100 \quad (1)$$

Determination of the Degree of Amidation by FT-IR

The degree of amidation (DA) was determined using Eq. 2 by analyzing the integrated area under the bands corresponding to the amidated groups in the infrared spectra and the total number of carboxylic groups. The integrated areas were calculated using Origin 8 software (Šimkovic et al. 2009).

$$\%DA = \frac{area\ of\ amidated\ carboxylic\ groups}{area\ of\ carboxylic + area\ of\ amidated\ carboxylics} \times 100 \quad (2)$$

Determination of Molar Mass by Gel Permeation Chromatography

The Gel Permeation Chromatography (GPC) technique was used to verify, among other possible factors, the average molecular mass and polydispersity of pectin molecules. Gel permeation chromatography analysis for the sample was performed on a Shimadzu LC-10AD chromatograph with an RID-10A refractive index detector at 40 °C. The column used had the following characteristics: linear ultrahydrogel 7.8 × 300 mm, mobile phase of $NaNO_3$ 0.1 mol/l at room temperature, flow of 1 ml/min, and the volume of of 20 µl of injected sample. To determine the molar masses of the analyzed samples, pullulan standards (Shodex Denko®) were used (MM from 5.9 × 103 to 7.88 × 105 g/mol).

Animals

Adult, wild zebrafish, both sexes aged 60–90 d, with sizes of 3.5 ± 0.5 cm and weight of 0.4 ± 0.1 g, were obtained from Agroquímica: Comércio de Produtos Veterinários Ltda., a supplier in Fortaleza (Ceará, Brazil). Groups of 60 fish were acclimated for 24 h in glass aquariums (30 × 15 × 20 cm) containing dechlorinated water (Protec-Plus®) and air pumps with submerged filters at 25 °C and pH 7, with a circadian cycle of 14:10 h of light: dark. The fish were fed *ad libitum* for 24 h prior to the experiment. After the experiments, fish were euthanized using cold water (5 °C). All experimental procedures were approved by the Ethics Committee on Animal Use of the State University of Ceará (CEUA-UECE), under protocol number 7210149/2016.

Acute Toxicity Against Adult Zebrafish

As an adaptation of the method, zebrafish (n = 7/group) was treated intraperitoneally with 20 µl of samples (4, 20, and 40 mg/kg), diluted in 3% DMSO. As a negative control, 3% DMSO was used. After 24, 48, 72, and 96 h, the values obtained with the number of dead zebrafish were subjected to statistical analysis to estimate the lethal dose to kill 50% (LD_{50}) zebrafish using the mathematical method Trimmed Spearman-Karber with 95% confidence intervals (Arellano-Aguiar et al. 2015).

Locomotor Activity Evaluation (Open Field Test)

An open field test was performed to assess whether the samples promoted changes in the motor coordination of the animals through sedation and/or muscle relaxation (Magalhães et al. 2017). The animals (n = 6/group) were treated intraperitoneally (*i.p.*) with 20 µl of the sample in doses: 4, 20, and 40 mg/kg. The vehicle (DMSO 3%) and diazepam (DZP; 4 mg/kg) groups were also analyzed. After 30 min of treatment, the animals were added to Petri dishes containing the same aquarium water and marked with quadrants. The locomotor activity was analyzed by counting the number of line crossings over a period of 5 min. Animals that did not receive treatment (naïve) were considered baseline (no change in locomotor activity).

Anxiolytic Assessment

Anxiety was observed using a light/dark test. Similar to rodents, zebrafish naturally avoid illuminated areas (Gonçalves et al. 2020). The experiment was conducted in a glass aquarium (30 cm × 15 cm × 20 cm) divided into light and dark areas. The aquarium was filled to 3 cm with dechlorinated tap water, which simulated a new shallow environment different from the conventional aquarium and was capable of inducing anxiety behaviors. In animals (n = 6/group), 20 µl of PECLG was administered *i.p.* at doses of 4, 20, and 40 mg/kg. The negative and positive control groups consisted of 3% DMSO and 4 mg/kg diazepam solution, respectively. After 1 h, the animals were placed individually in the light zone, and anxiolytic effects were measured based on the time spent in the light zone of the aquarium within 5 min of observation (Gebauer et al. 2011).

Assessment of GABAergic Neuromodulation

The GABAergic neuromodulation involved in the anxiolytic effect of the peptide was identified through pre-treatment with flumazenil (a GABAA antagonist) before the light/dark test (Benneh et al. 2017). Zebrafish (n = 6/group) were pretreated with flumazenil (4 mg/kg; 20 µl, *i.p.*). After 15 min, the most anxiolytic-effective dose of PECLG (4 mg/kg; 20 µl, *i.p.*) was administered. DMSO (3%) (vehicle; 20 µl *i.p.*) was used as a negative control. After 1 h of treatment, the animals were subjected to the light/dark tests.

Glucose Immersion-Induced Hyperglycemia

Animals (n = 6/group) were placed in a D-glucose solution at 111 mM/l (Capiotti et al. 2014) in antichlorinated water in 10 l glass aquariums (30 × 15 × 20 cm) for 7 days and kept at room temperature. The power schedule and general

maintenance procedures were described in the previous section. The glucose solution was changed daily to avoid contamination with opportunistic microorganisms.

To verify whether the effects caused by treatment with a 111 mM glucose solution would persist, on the 8th day, the animals were removed from the glucose solution and kept in water without chlorine until the 11th day. The aquarium water was changed daily throughout the experiment.

Determining Blood Glucose Levels

For all tests involving the determination of blood glucose levels, before starting the blood collection procedures, the fish were fasted for 12 h and subsequently euthanized in an ice bath (2 °C) to perform the glycemic reading on days 8 and 11. Immediately, the tail was clipped, and blood glucose readings were taken by placing a glucometer test strip (Accu-Check Active) directly on the clipped tail.

Hyperglycemia Treatment

After the induction of hyperglycemia, from the 8th day, the animals (n = 6/group) were treated for 4 consecutive days with PECLG (40 mg/kg; 20 µl, *p.o.* each), control (3% DMSO), and metformin (200 mg/kg; 20 µl, *p.o.*). A group of animals was kept untreated (naïve). Twelve hours after the last treatment, the fasted animals were euthanized in an ice bath to determine their glycemic levels, as mentioned above.

Molecular Docking

The two-dimensional chemical structures of pectin were generated using plugins available in MarvinSketch software (https://chemaxon.com/products/marvin). Structural optimization was performed using Avogadro's code (Hanwell et al. 2012), which was configured to employ the force field Merck Molecular Force Field 94 (MMFF94) (Halgren 1996). The optimization was carried out using the Steepest Descent algorithm (Meza 2010), with specific parameters including 500 numerical steps and a convergence parameter of 10 e^{-7} (da Silva et al. 2020).

The *C*-terminal subunit of maltase-glucoamylase (CtMGAM) was selected from the Protein Data Bank—RCSB protein database (https://www.rcsb.org/). The enzyme structure was resolved by X-ray diffraction, and the resulting pattern had a a resolution of 2.88 Å (R-Value Free: 0.284, R-Value Work: 0.218, and R-Value Observed: 0.222). As a prerequisite for preparing the protein structure, water molecules were removed, while Gasteiger charges and essential hydrogen atoms were added (Yan et al. 2014).

The AutoDock Vina code was used for docking simulations (Trott and Olson 2009). The grid box was centralized to encompass all protein chains with the dimensions (x, y, z) = (-45,828, 21,487, 17,927) and parameters 126 Å 82 Å 124 Å. Fifty independent simulations were performed, obtaining 20 poses for each. For statistical validation of the simulations, redocking procedures were performed, and RMSD (Root Mean Square Deviation) values were evaluated. The parameter for choosing the best pose values was less than 2 Å and the free binding energy (ΔG) was less than 6 kcal/mol. The results were analyzed and visualized using the Discovery Studio Visualizer and UCSF Chimera codes (Biovia et al. 2016; Pettersen et al. 2004).

Statistical Analysis

Results were expressed as mean values ± SEM for each group of six animals. After confirming the normal distribution and homogeneity of the data, the differences between the groups were subjected to a one-way analysis of variance (ANOVA), followed by Tukey's test. All analyses were performed using GraphPad Prism 6.0 software. The level of statistical significance was set at 5% ($p < 0.05$).

Results and Discussion

Extraction and Characterization of Pectin

After pectin extraction from Galician lemons, the yield was calculated using the following formula:

$$\%Y = \frac{Lyophilized\ PECLG}{Lyophilized\ Galician\ lemon\ rinds} \times 100$$

The yield was calculated to be 15.94%.

The amount of pectin extracted from the Galician lemon agrees with the literature, as the extraction conditions and the variation in the botanical origin directly affect not only the extraction yield, but also the physicochemical characteristics of the pectin (Canteri et al. 2012). The yield varies quantitatively according to the source of raw material used, which is between 7.6 and 26% for tangerines, 15 and 25% for lemons, and 5 and 30% for oranges (Venzon et al. 2015; Colodel et al. 2018).

Structural Characterization

The analysis of the infrared spectrum (Fig. S1) allowed for the identification of a broad and strong band at 3304 cm^{-1}, corresponding to the stretching of hydroxyl groups (O–H). This stretching is associated with the inter- and intramolecular structures of the galacturonic acid units present in pectin. Additionally, the analysis revealed a possible

overlap in the 3300 cm^{-1} region, which is a characteristic of the N–H amide stretching (Liang et al. 2020). The band present in the region of 2151 cm^{-1} characterizes the N–H stretching of ammonium carboxylate (Pavia et al. 2010; Fajardo et al. 2012). The vibrational bands corresponding to 1747 cm^{-1} and 1602 cm^{-1} are characteristic of esterified carboxylic and carboxylate groups, respectively. The band at 1602 cm^{-1} is accompanied by a signal at 1428 cm^{-1}, corresponding to the symmetric stretching of the carboxylate group, overlapping with the stretching at 1400 cm^{-1}, which corresponds to the primary amide C-N bond (Pavia et al. 2010). Amide carbonyls (1680 cm^{-1}–1620 cm^{-1}) partially overlapped with the N–H fold (1640 cm^{-1}–1620 cm^{-1}), favoring the emergence of a doublet in the carbonyl region (Pavia et al. 2010). The bands that characterized the main functional groups of pectin extracted from Galician lemon (PECLG) are shown in Fig. S2.

The PECLG NMR ^{1}H spectrum (Fig. S3) reveals that the signals related to the anomeric hydrogens of the methyl esters of galacturonic acids are located in the region of 5 ppm, whereas the signals representing the neighboring hydrogen of galactopyranosyl carboxylate groups are located in the 4.96 ppm region (Rosenbohm et al. 2003; Synytsya 2003; Tamaki et al. 2008). The chemical shift at 4.56 ppm corresponds to the N–H of the amide group (Fajardo et al. 2012). The signal at 3.97 ppm corresponds to the methyl groups of esterified galacturonic acids, while the signal at 3.58 ppm refers to the hydrogen neighboring the chain-binding oxygen, which suggests the presence of mannose residues (Rosenbohm et al. 2003; Synytsya 2003).

The ^{13}C NMR spectrum of the pectin (Fig. S4) shows a main signal at δ 175,136 ppm corresponding to the amidated carbon carboxyl groups of galacturonic acid. The main signals of the galacturonic acid carbons were observed at δ 78.532, 74,046, 71,662, 69,346, and 68,733 ppm. The signals at 99,480, and 22,834 correspond to rhamnose units. These data are compatible with those of other ^{13}C NMR studies (Synytsya 2003; Fracasso et al. 2018).

Based on the calibration curve of the pullulan polysaccharide standards, the chromatogram in Fig. S5 illustrates the polydispersity of the pectin under study. The polydispersity index analyzes the arrangement of molecular masses based on the width of the graph. PECLG was found to present a polydisperse character.

The values referring to the polydispersity index are calculated as the ratio between the weight-average molecular mass (Mw) and the numerical average molecular mass. This index quantifies the variation in masses, with values equal to 1.0 indicating monodispersity and values greater than 1.0 indicating polydispersity; the further from 1.0, the greater the variation (Zanella and Taranto 2015). According to the results, PECLG presents a variation in chain size and can be classified as polydisperse because of its polydispersity of 2.9.

The molecular mass obtained for was 137,161 g/mol. A study carried out with orange pectin using citric acid and nitric acid as extracting solvents obtained pectins with a high degree of esterification, with molar masses ranging from 83,486 to 138,787 g/mol and a polydispersity equal to 3.0 (Venzon et al. 2015).

Petkowicz et al. (2017) extracted pectin from fresh and freeze-dried watermelons using nitric acid as an extracting solvent and obtained molar masses of 34,510 and 40,390 g/mol and polydispersity values of 3.2 and 4.6, respectively. Pectin extracted from passion fruits using citric acid as an extracting solvent had a molar mass of 116,000 g/mol (Canteri et al. 2012).

The molecular mass of pectin directly interferes with its gel formation ability, a crucial property that determines its intended use. A high molecular weight guarantees the formation of gels under different conditions, whereas a low molecular weight hinders gelation. The values reported by the authors cited in this paper obtained a lower mass than that found in this study. This disparity can be attributed to the different extraction methods and the plant source used, as reported by Picot-Allain et al. (2022).

Determination of the Degree of Esterification and Amidation

After analyzing the infrared spectrum, the formation of amidated pectin was observed. The calculations determined that the pectin had a degree of esterification equal to 14.43% and an amidation degree equal to 37.26%, and it is thus classified as an amidated pectin with a low degree of esterification. Liang et al. (2020) performed a reaction between a citrus pectin, characterized by a low degree of esterification, and different concentrations of ethylenediamine to obtain amidated pectins. The pectinsobtained had different degrees of amidation (31.62, 38.94, 43.13, and 48.31%). In order to extend the functionality of natural polymers, Chen et al. (2020) performed a reaction between a pectin with a high degree of esterification and different amino acids. The reactions carried out yielded pectins of varying degrees of esterification and a degree of amidation equal to 6.5%, as calculated from infrared and NMR ^{1}H spectra. Therefore, the pectins reported in the aforementioned studies were considered to have a low degree of amidation, a characteristic that aligns with the pectin evaluated in this study.

Acute Toxicity Against Adult Zebrafish

Adult zebrafish have been used as an alternative animal model for rodents in genetic, developmental, neurobiological, and toxicological tests (Resende 2016) because of the low cost involved and their diverse adaptability, short reproduction cycles, high fecundity, and transparent embryos (Dai

et al. 2014). Its small size in adulthood leads to a decrease in the amount of substances to be examined and administered, as well as a reduction in the amount of reagents and materials used in the treatment and maintenance of animals (Hill et al. 2005). It is important to highlight that adult zebrafish are also used to assess the toxicity of pharmaceutical compounds (Hill et al. 2005) as well as for toxicological biomonitoring in drug development (Caballero and Candiracci 2016). Pectin proved to be safe, as it did not show toxicity in adult zebrafish during the 96-h analysis ($LD_{50} > 40$ mg/kg). In this study, the adult zebrafish was used as an animal model to assess the acute toxicity of the PECLG sample. Similar results were found in pectins from other species. Edirisinghe et al. (2019) carried out a trial with larvae and an adult Zfa model and showed that pectins extracted from *Spirulina maxima*, used as immunomodulatory agents, did not show toxicity. The toxicity of red ginseng extract modified with pectin as a dietary supplement was assessed in a research, which found no evidence of acute toxicity in rats throughout the conducted tests (Park et al. 2019). According to Jonken et al. (2020), the pectin extracted from carrots is non-toxic.

Glucose Immersion-induced Hyperglycemia

Zebrafish are promising organisms to study hyperglycemia and its complications (Gleeson et al. 2007; Capiotti et al. 2014). Similar to rodents, a hyperglycemic state can be induced in zebrafish through the destruction of pancreatic beta cells (Moss et al. 2009; Shin 2012). Another protocol involves immersing zebrafish in a glucose solution, based on the assumption that fish can easily absorb water molecules and regulate their internal concentrations of water and total solutes (Gleeson et al. 2007; Santos et al. 2018; Capiotti et al. 2014). In zebrafish, glucose uptake/capture occurs through the glucose transporter, GLUT, which is expressed in the gills and intestine (Tseng et al. 2009).

The results showed that after the 4th day of glucose withdrawal, a continuous increase in blood glucose levels in the treated animals occurred compared to the untreated group (****$p < 0.0001$ vs. naive) (Fig. 1). These data show that hyperglycemia can be induced during the seven days of treatment and maintained until the eleventh day in adult zebrafish. During the four days of treatment, pectin reduced (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control) the hyperglycemia of animals, an effect similar to that of metformin (positive control). Thus, pectin reduced the blood glucose content of the animals to basal levels (< 100 mg/dl) ($p > 0.05$) (Fig. 2). The results demonstrated that PECLG reduced hyperglycemia in animals (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control), an effect similar to that of metformin (positive control) (Fig. 3).

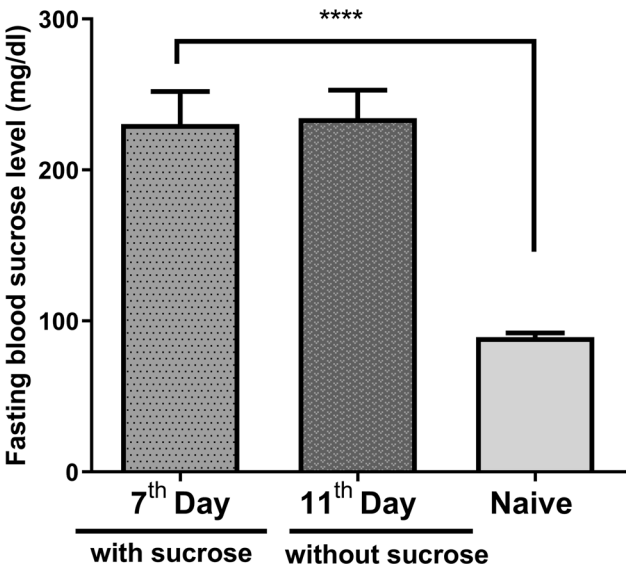

Fig. 1 Effect of sucrose elimination (83.25 mM) in hyperglycemic zebrafish. Naive group—untreated animals. One-way ANOVA followed by the Tukey test: (****$p < 0.0001$ vs. Naive)

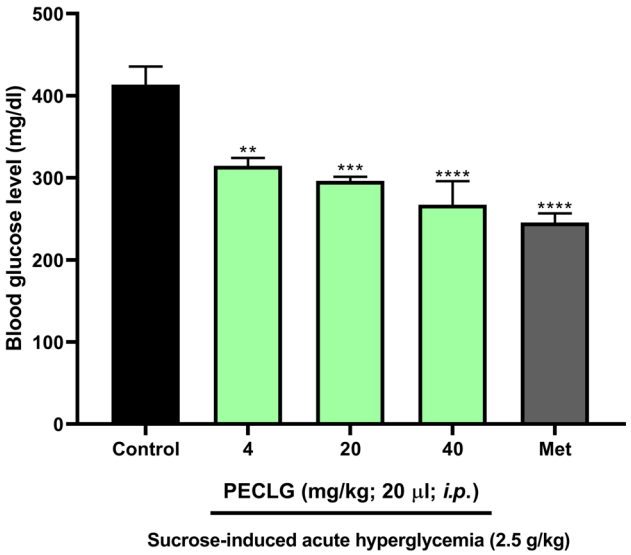

Fig. 2 Effect of pectin PECLG (4, 20, 40 mg/kg, *p.o.*) on acute hyperglycemia induced by sucrose (83.25 mM). Control—(3% DMSO); Met – metformin (200 mg/kg, *p.o.*); Naive group—untreated animals. One-way ANOVA followed by the Tukey test: (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control)

When using zebrafish to determine the hypoglycemic potential of pectin, a method of immersion in a glucose solution was used, and a result similar to that of metformin was observed. Wu et al. (2017) performed tests using pectins from the fruit *Ficus pumila* L., Moraceae, and obtained positive results for the treatment of hyperglycemia in diabetic mice. Following the same line of research, Zhan et al.

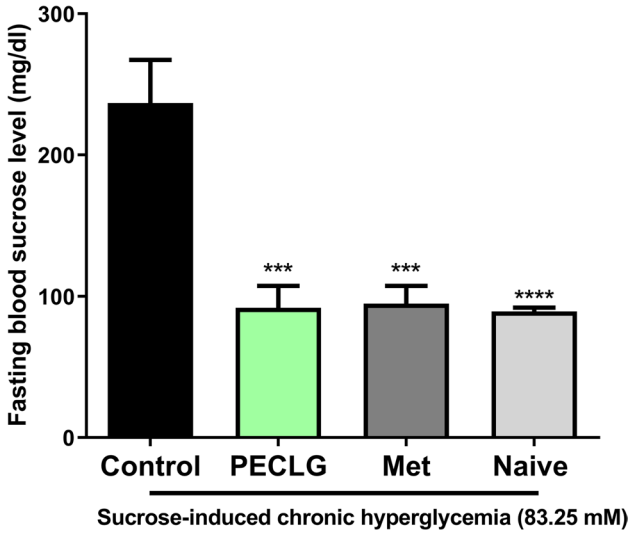

Fig. 3 Effect of PECLG (40 mg/kg; *p.o.*) on sucrose-induced hyperglycemia (83.25 mM). Control—(3% DMSO); Met – metformin (200 mg/kg; *p.o.*); Naive group—untreated animals. One-way ANOVA followed by the Tukey test: (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control)

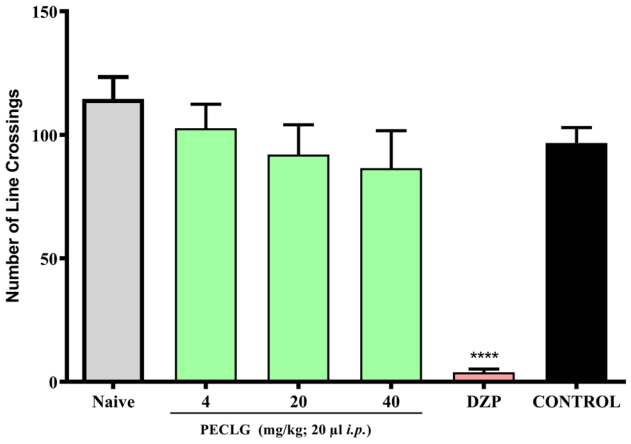

Fig. 4 Fig. Effect of PECLG on the locomotor activity of adult zebrafish (*Danio rerio*) in the Open Field Test (0–5 min). Naive—untreated animals. DZP – diazepam (4 mg/kg; 20 µl, *i.p.*). Control—3% DMSO (20 µl *i.p.*). Values represent mean ± SEM for 6 animals/group. One-Way ANOVA followed by Tukey's Test (***$p < 0.001$; vs. control)

(2019) used commercial citrus fruit pectin with a high degree of esterification to investigate obesity induced by a typical environmental pollutant. On analyzing the nutritional parameters of mice, they realized that pectin was able to significantly reduce fasting blood glucose levels, suggesting that pectin prevented hyperglycemia.

The impact of the degree of esterification on several biological processes has been reported in previous studies (Samout et al. 2016; Eliaz and Raz 2019). Lemon pectins, for example, with low degree of esterification content, can potentially be applied in the prevention and control of diabetes, protecting β cells against inflammatory and oxidative stress. This substantiates the correlation between higher consumption of dietary fiber and a reduced incidence hyperglycemia (Hu et al. 2020).

Assessment of Locomotor Activity (Open Field Test) and Anxiolytic Evaluation

The assessment of locomotor activity serves as a behavioral analytic parameter used to evaluate the action of drugs that have the potential to affect the central nervous system of adult zebrafish and cause locomotor impairment (Kurta and Palestis 2010; Gebauer et al. 2011; Taylor et al. 2017). This activity can be explored through an open field test in an aquarium as well as in petri dishes (Ahmad and Richardson 2013). Different parameters, such as freezing (immobility), can be evaluated. The natural behavior of zebrafish in the open field is characterized by constant swimming activity, and manifestations of immobility are rarely observed in their natural conditions (Cachat et al. 2011).

The analysis of locomotor activity explored in an open field can be used to evaluate hyperactivity, an indicative of anxiety. Treatment of zebrafish with anxiolytic drugs such as benzodiazepines can increase exploratory activity in the open field (Cachat et al. 2011) or cause sedative effects and decrease locomotor activity (Benneh et al. 2017).

The open field test was adapted to evaluate the locomotor activity of adult zebrafish under the action of analgesic drugs (Magalhães et al. 2017). The same method was used with the samples given to evaluate actions on the locomotor system of the zebrafish. As a result, it was observed that the PECLG sample did not change the animals' locomotion during the 5-min analysis (Fig. 4).

Medications used to treat anxiety, such as benzodiazepines (anxiolytic drugs), have a sedative action, in turn decreasing locomotor activity (mobility) in adult zebrafish in the open field, as highlighted by Benneh et al. (2017). In contrast, PECLG did not change the locomotion of zebrafish and presented an anxiolytic action, as evidenced by zebrafish remaining longer in the light zone of the aquarium when compared to DZP.

The results demonstrated that PECLG (4, 20, and 40 mg/kg) increased (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control) the time spent by the animals in the light region of the aquarium in the light/dark test (Fig. 5), demonstrating an anxiolytic effect similar to that of DZP (4 mg/kg), the positive control. The lowest dose of PECLG (4 mg/kg) was the most effective in promoting the anxiolytic behavior of the animals, as they remained in

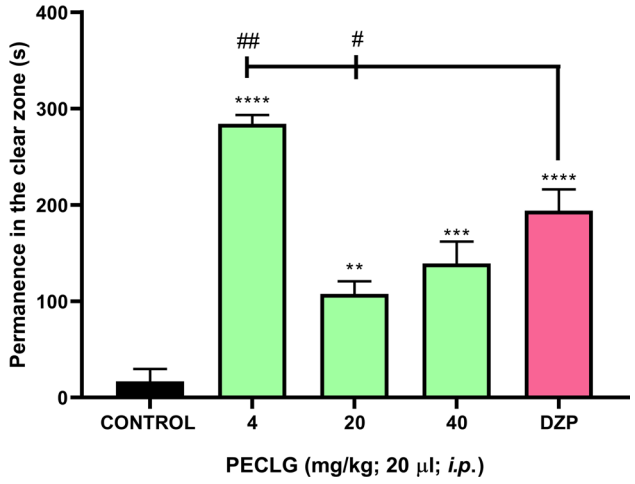

Fig. 5 Effect of PECLG on zebrafish anxiety in the light/dark test (0–5 min). Values represent the mean ± SEM for 6 animals/group; One-way ANOVA followed by Tukey's test for the light/dark test. Asterisks indicate statistical significance compared to the control group (control – 3% DMSO), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. control, and the hash sign indicates statistical significance compared to the Diazepam (DZP) treated group, $\#p < 0.05$, $\#\#p < 0.01$ vs. DZP

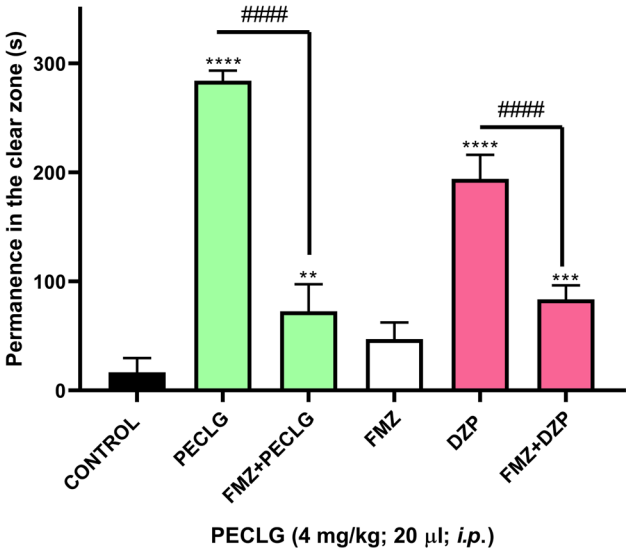

Fig. 6 Effect of flumazenil on the anxiolytic behavior of animals treated with PECLG (4 mg/kg) in the light/dark test. Values represent the mean ± SEM for 6 animals/group; Two-way ANOVA followed by Tukey's test for mechanism of action. Asterisks indicate statistical significance compared to the control group (control – 3% DMSO), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. control; and the hash sign indicates statistical significance compared to the group treated with antagonist (Flumazenil—Fmz) $\#\#\#\#p < 0.0001$ vs. Fmz + PECLG

the light area of the aquarium most of the time, surpassing the effects observed with DZP.

The investigation into the mechanism by which anxiety is mediated via GABAergic neurotransmission included the administration of flumazenil as apre-treatment. The anxiolytic behavior induced by PECLG at a dose of 4 mg/kg was significantly reduced ($\#\#\#\#p < 0.0001$ vs. Fmz + PECLG; Fig. 6) in the presence of flumazenil. This was evidenced by the increased time spent by the fish in the dark region of the aquarium, indicating anxiety behavior and demonstrating that the anxiolytic effect of PECLG is related to the GABAA receptor.

Several studies have used zebrafish as an animal model to evaluate diseases related to the central nervous system (CNS) by analyzing anxiolytic, convulsive, memory, and learning activities using various types of bioactive compounds, both synthetic and natural (Ferreira et al. 2021; Luchiari et al. 2021; Silva et al. 2021; Da Silva et al. 2022).

Molecular Docking

Molecular docking simulations are one of the most used tools to aid the design of modern medicines. Thus, to understand the interaction of pectin in the hypoglycemic process, molecular docking studies were performed. To statistically validate the outcomes of the simulations pertaining to complex formation and the determination of the most thermodynamically stable conformation (referred to as the best pose), the RMSD (Root-Mean Square Deviation) metric was employed. The RMSD was calculated based on the measurement of the average distance between the atoms of the two ligands, with validation criterion values close to 2 Å (Yusuf et al. 2008). All docking and redocking simulations had values lower or close to 2 Å.

To obtain a structural view of the mechanism of CtMGAM inhibition, the modes of the ligand at the active site were investigated using a computer simulation technique (molecular docking) (Duong et al. 2020). Using affinity energy as a parameter, it can be concluded that pectin has a higher affinity than α-acarbose and metformin because it has a lower affinity energy (-8.0 kcal/mol). This analysis can be refined when evaluating the inhibition constants (Ki) and pKi. It can be inferred that the lower the Ki value and the higher the pKi, the greater the affinity of the ligand for the protein, and consequently, the lower the concentration of the ligand required to inhibit enzymatic activity (Kadela-Tomanek et al. 2021).

Highlighting the "best pose" of the pectin-CtMGAM complex (Fig. 7), the pectin simulation presented a RMSD value of 1,878 Å. The formed complexes presented affinity energy ranging from -7.8 to -8.0 kcal/mol, with the exception of the metformin ligand, which, by the redocking technique, presented an energy of order -5.2 kcal/mol (Table S1). Regarding the interactions involved in the formation of the

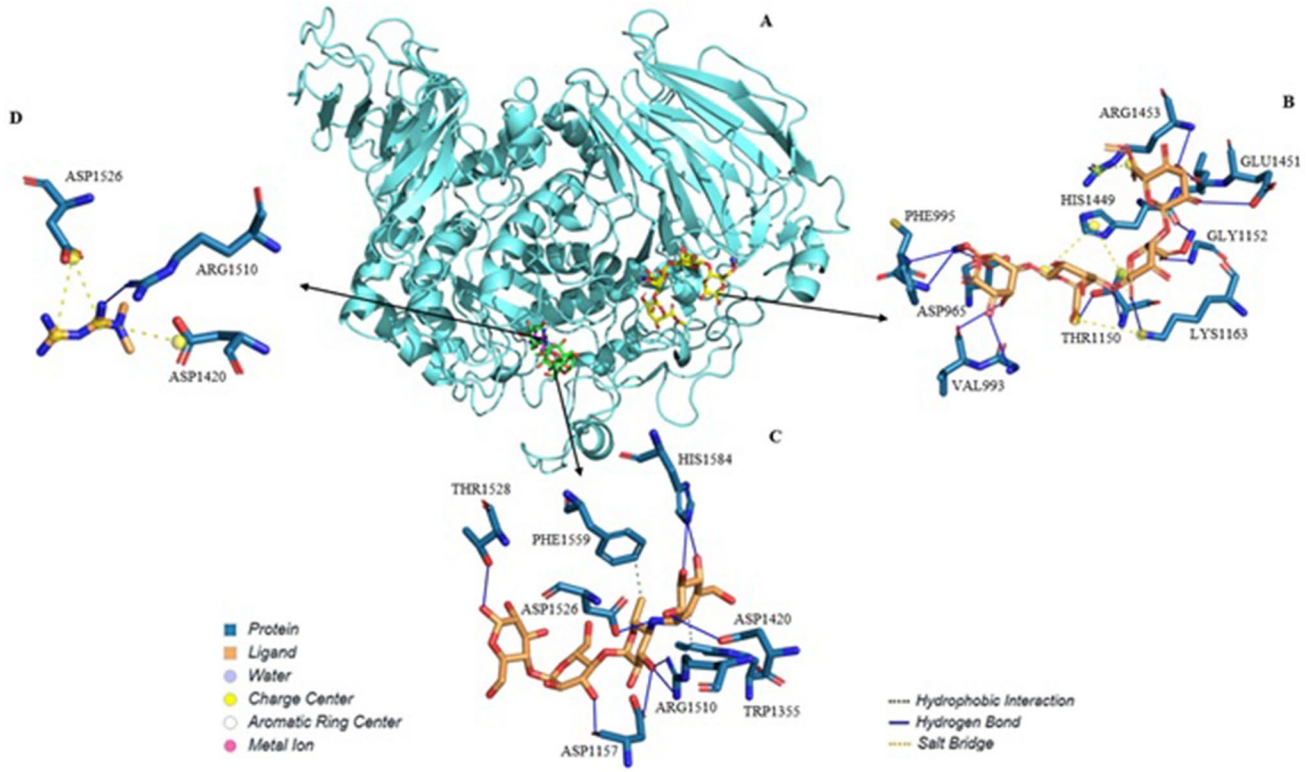

Fig. 7 Interaction complex of CtMGAM enzyme with ligands (**A**); Interaction maps of pectin ligand (**B**), co-crystallized alpha-acarbose inhibitor (**C**) and metformin receptor drug (**D**)

ligand–protein complex, the pectin-CtMGAM complex showed seventeen interactions (twelve H-bonds and five salt bridges), whereas the α-acarbose-CtMGAM complex showed twelve interactions (two hydrophobic and ten H-bonds), and the metformin-CtMGAM complex showed six interactions (two H-bonds and four salt bridges), as observed in Table S2.

From this perspective, it can be asserted that pectin will need a lower concentration to inhibit the CtMGAM enzyme, as it presented a lower Ki (1.358×10^{-6}) and a higher pKi value (5.86) compared to the co-crystallized inhibitor (α-acarbose) and the reference drug (metformin). This indicates the inhibitory potential of pectin on CtMGAM and validates the results obtained in the experimental procedures.

By analyzing the formation of complexes, it becomes evident that pectin is complexed in a different site from α-acarbose and metformin, both of which complex in the same region (Fig. 7). This observation suggests the potential for a synergistic impact when combined withdrugs. However, it can be observed that the pectin-CtMGAM complex is predominantly formed by hydrogen bonds, where the hydrogen and nitrogen atoms allow the formation of eleven strong bonds, with bond lengths varying between 2.06 and 3.06 Å, and only a moderate one in the order of 3.44 Å involving residue GLN1152A. It is noteworthy that, in addition to hydrogen bonds, it is possible to observe five saline bridges with distances varying between 4.21 and 5.06 Å.

The profile of the pectin-CtMGAM complex is very similar to that of the α-acarbose-CtMGAM, which, despite complexing at different sites, has a predominant formation profile through hydrogen bonds formed by the contribution of oxygen and nitrogen atoms, predominantly complexing with basic amino acid residues like arginine, histidine, and lysine and polar ones like tyrosine, asparagine, glutamine, and tyrosine.

Conclusion

Analysis of spectroscopic data confirmed the structure of amidated pectin, and toxicity tests indicated that the compound was not toxic to adult zebrafish during the 96 h of analysis. Regarding its pharmacological potential, pectin has been shown to reverse hyperglycemia in zebrafish with a possible synergistic effect when combined with metformin (reference drug) and acarbose (reference inhibitor), as confirmed by molecular docking studies. In addition, pectin demonstrated anxiolytic effects via GABAergic neurotransmission, like the positive control, DZP.

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Authors' Contributions The authors declare individual contribution to the article as follows: data collection, analysis, and manuscript preparation: AMBA; conception of the study: NAPP and AEQRC; data interpretation: SMCS; supervision of manuscript writing: MKAF and AWS; assistance with zebrafish data and analysis: LM; contribution to the methodology of the study: ESM and MMM; in silico analysis: IGPV; review and editing: HSS; supervision; JESAM; guidance throughout the research process: II. The completed manuscript underwent a comprehensive assessment by all authors, resulting in approval for its publication.

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Data Availability Data is available on request. Please contact helcio_santos@uvanet.br.

Declarations

Ethics Approval and Consent to Participate The work was approved by the Ethics Committee on the Use of Animals of the State University of Ceará (CEUA-UECE; nº 04983945/2021) following the Ethical Principles of Animal Experimentation.

Competing Interests All authors have no conflicts of interest to declare with respect to the research, authorship, and/or publication of this work.

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