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Inhibiting enzymatic starch digestion by hydrolyzable tannins isolated from *Eugenia jambolana*

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ABSTRACT

Slowing down starch digestion is one method of controlling postprandial hyperglycaemia of diabetes, for which naturally occurring α-amylase inhibitors from edible botanicals have a great potential. We reported herein that *Eugenia jambolana*, a traditional herbal tea for the treatment of diabetes in South Asia, contains potent α-amylase inhibitors because of monomeric and polymeric hydrolyzable tannins (HT). These compounds demonstrated a dose dependent inhibitory activity against α-amylase ($IC_{50} = 1.1 \pm 0.4$ μg/mL), which was significantly stronger than acarbose ($IC_{50} = 19.0 \pm 2.0$ μg/mL). Kinetic studies revealed that the HT were mixed non-competitive inhibitors against α-amylase. Using an *in vitro* human starch digestion model, incorporation of 0.125 mg/mL HT into a real food system (wheat flour) was effective in delaying enzymatic starch digestion moderately, with a significantly stronger inhibitory effect in the absence of proteins in the food matrix. Pre-incubation of HT with α-amylase prior to substrate addition also significantly enhanced their inhibitory activity. These results provide useful knowledge on HT as potential α-amylase inhibitors, which could potentially alleviate postprandial hyperglycaemia in diabetic patients.

1. Introduction

Type 2 diabetes is a chronic disease affecting an estimated 346 million people worldwide, who suffer from chronic hyperglycaemia due to pancreatic β-cell dysfunction and/or increased resistance to insulin (Danaei et al., 2011). The search for safe and effective treatment methods to combat this growing epidemic has uncovered botanicals as a valuable source of starch hydrolase inhibitors. These inhibitors exert their anti-diabetic effect by hindering the activity of starch hydrolyzing enzymes present in the small intestinal brush border, such as α-amylase and α-glucosidase. α-Amylase (EC 3.2.1.1) catalyses the initial hydrolysis of starch into shorter oligosaccharides and disaccharides, through the cleavage of α-D-(1,4) glycosidic linkages between glucose residues. Inhibitors of α-amylase would thus effectively delay starch digestion. This would reduce the rate of glucose absorption into the bloodstream and consequently blunt postprandial plasma glucose increase in diabetic patients.

Food-grade botanicals, particularly traditionally used herbal teas, could be ideal candidates as alternative hypoglycaemic agents, as they provide a rich source of bioactive ingredients that may potentially act as starch hydrolase inhibitors (Devalaraja, Jain, & Yadav, 2011; Patel, Kumar, Laloo, & Hemalatha, 2012). One such potential plant is *Eugenia jambolana* (EJ), an evergreen tree distributed in the Indian sub-continent and Southeast Asian countries. In the Ayurvedic system of the subcontinent, the entire plant of EJ, including its seed, fruit, leaves, flower and bark, has been being widely used as herbal teas for the treatment of diabetes (Chhetri, Parajuli, & Subba, 2005). Continuous treatment of ethyl acetate and methanolic extracts of EJ seed extracts (200 mg/kg of body weight) for a period of 15 days had produced a significant decrease in the blood sugar levels of streptozotocin-induced diabetic rats (Kumar et al., 2008). Likewise, oral administration of its bark extracts daily over a longer period of 45 days (300 mg/kg of body weight) had significantly decreased blood glucose levels and increased levels of plasma insulin and C-peptide of both normal and diabetic rats (Saravanan & Leelavinothan, 2006).

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Despite its potent anti-diabetic effects as demonstrated in *in vivo* animal studies, there is limited scientific knowledge on the bioactive chemical constituents of the EJ bark extract. Preliminary studies showed that crude stem bark extracts of the plant were strong α-amylase inhibitors. Therefore, the aim of this study was to evaluate the α-amylase inhibitory effect of EJ bark extract and to isolate and characterize the compounds responsible for its inhibitory activity in wheat starch and wheat flour.

2. Materials and methods

2.1. Reagents and instruments

α-Amylase (type VI-B, from porcine pancreas), porcine stomach pepsin powder, corn starch, acarbose and cellulose membrane dialysis tubing were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Anhydrous sodium carbonate was purchased from Thermo-Fisher (Singapore). Wheat flour (Prima Flour) was obtained from Prima Ltd. (Singapore). Wheat starch was obtained from Yiak Say Hang Food Industries Pte Ltd (Singapore). Liquid chromatography and mass spectra were acquired using a Bruker Amazon ion trap mass spectrometer (Billerica, MA) equipped with a Dionex ultimate 3000RS HPLC system (Bannockburn, IL). The column used was a 250 mm × 4.6 mm i.d., 5 μm, Develosil diol with a 4 mm × 4 mm i.d. guard column of the same materials (Seto, Japan). The heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 5.5×10^5 Pa for sheath gas flow rate and 1.4×10^5 Pa for auxiliary gas flow rate. The full scan mass spectra from *m/z* 50 to 2000 were acquired in negative ion mode with a scan speed of 1 s^{-1}. The MS^n collision gas was helium with collision energy of 30% of the 5 V end-cap maximum tickling voltage. The Synergy HT microplate reader of Biotek Instruments Inc. (Winooski, VT, USA) was used to measure starch digestion kinetics.

2.2. Extraction and fractionation of HT

EJ bark (100 g) was mixed with methanol (80%, 3 × 1 L) and shaken for 4 h and filtered under reduced pressure. The filtrate was subjected to rotary evaporation to remove the solvent. The solid crude extract obtained was suspended in 500 mL water and was successively extracted with chloroform, ethyl acetate, butanol and water. Each fraction was measured for its amylase inhibition activity. The butanol fraction exhibited highest inhibitory activity and was subjected for further fractionation.

The butanol fraction (0.8 g) was loaded on a Sephadex LH-20 column (column i.d., 5 cm) containing 50 g of LH-20 equilibrated with methanol/water (1:1) for 4 h. The column was eluted with H_2O (Fraction 1), methanol (Fractions 2–3), methanol/acetone (4:1, v/v) (Fractions 4–7) and methanol/acetone (1:1, v/v) (Fractions 8–9). The solvents from each fraction ware removed on a rotary evaporator at 40 °C and the resulting residue was freeze-dried to give a dark brown powder. Each fraction was tested for α-amylase inhibitory activity. Fractions 5 (B5) and 9 (B9) exhibited the strongest inhibitory activity. Therefore, further characterization of these two fractions was conducted by HPLC and $LC–MS^n$.

2.3. Acid hydrolysis of tannins

Acid hydrolysis of compounds in B5 and B9 was performed as described by Oszmianski, Wojdylo, Lamer-Zarawska, and Swiader (2007). The compounds (5 mg) were hydrolyzed with hydrochloric acid (2 M, 2 mL) in a boiling water bath for 1 h. After cooling, sodium hydroxide (2 M, 2 mL) and methanol (6.0 mL) were added to the vial. The slurry was sonicated for 20 min with occasional shaking and centrifuged at 10,000 *g*. The supernatant was tested for α-amylase inhibitory activity and analyzed via HPLC and $LC–MS^n$.

2.4. α-Amylase inhibition assay

α-Amylase inhibitory activity was determined following a reported method (Liu, Song, Wang, & Huang, 2011). In a 96-well microplate, 20 μL of enzyme solution was pre-incubated with 20 μL of the inhibitor solution with a series of concentrations from 0.8 to 2.0 μg/mL, in a microplate reader for 15 min at 37 °C. The reaction was initiated by injecting 60 μL of starch solution using a 12-channel multichannel pipette. The turbidity change was immediately monitored at 660 nm for 2 h. The shaking intensity of the microplate was set at the highest level to ensure sufficient mixing and to avoid starch sedimentation. The percentage of inhibition was defined by equation (1):

$$\%\ \text{inhibition} = \frac{AUC_{sample} - AUC_{control}}{AUC_{sample}} \times 100 \quad (1)$$

in which AUC_{sample} is the area under the curve (AUC) of the inhibitor; $AUC_{control}$ is the area under the curve without inhibitors. IC_{50} can be defined as the concentration of inhibitor that produces 50% inhibition of the enzyme activity under the specified assay condition and serves as an estimation of the strength of the inhibitory activity of each fraction. IC_{50} of each fraction was calculated by interpolation of percentage inhibition against the inhibitor concentration curve.

2.5. HPLC and tandem mass spectrometry

Fractions B5 and B9 were dissolved in methanol and filtered through a PTFE 0.45 μm membrane filter (Epsom) before injection into the LC/MS^n system. The elution conditions were as follows: flow rate, 1.0 mL/min; column temperature, 35 °C; mobile phase A, 2% acetic acid in acetonitrile; mobile phase B, acidic aqueous methanol (CH_3OH:H_2O:HOAc, 95:3:2 v/v/v). The starting mobile phase condition was 7% B holding isocratic for 3 min before ramping solvent B to 37.6% over 57 min and then to 100% B 3 min thereafter. B was held at 100% for 7 min prior to returning to starting conditions (7% B) in 6 min. The column was equilibrated with 7% B for 5 min prior to the next run.

Separation was performed on an Atlantis T3 reversed-phase C18 column (150 mm × 4.6 mm i.d., 3 μm particle size). Supernatants of hydrolyzed B5 and B9 were filtered through a PTFE 0.45 μm membrane filter before being injected into the LC/MS^n system. The elution conditions were as follows: flow rate was 0.2 mL/min; the mobile phase was composed of solvent A, 0.1% (v/v) acetic acid in water and solvent B, acetonitrile. The starting mobile phase condition was 5% B, ramping up to 100% over 30 min. B was held at 100% for 10 min prior to returning to starting conditions (5% B) in 3 min. The column was equilibrated with 5% B for 7 min prior to the next run.

2.6. Kinetic analysis

A mixture of α-amylase (0.648 U, 150 μL) and inhibitor solution (150 μL) of concentrations of 0.01, 0.015 and 0.02 mg/mL were incubated at 37 °C for 15 min. Starch solution (300 μL, 2 mg/mL) was added to each tube and incubated at 37 °C for 5 min. The reaction was terminated by addition of 100 μL of 3,5-dinitrosalicylic acid (DNSA) to the reaction mixture (100 μL) taken from each tube. The mixture was placed in a boiling water bath for 5 min and cooled to room temperature. After dilution, the absorbance was measured at 540 nm. The procedure was repeated for multiple