In vitro inhibitory effects of *Pithecellobium dulce* (Roxb.) Benth. seeds on intestinal α-glucosidase and pancreatic α-amylase

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Abstract

This study sought to assess and characterize the inhibitory action of methanolic extract of *Pithecellobium dulce* (*P. dulce*) seeds on α-amylase and α-glucosidase enzymes as well as to characterize compounds responsible for these activities. The methanolic extract was assessed for total phenolic, flavonoid and triterpenoids content by using Folin-Ciocalteu’s reagent, Aluminum chloride (AlCl₃) and Vaniline-perchloric acid assay, respectively. The methanolic extract was further quantified with respect to intestinal α-glucosidase (maltase and sucrase) and pancreatic α-amylase inhibition by glucose oxidase method and Dimitrosalicylic acid (DNSA) reagent, respectively. The IC₅₀ values of methanolic extract of *P. dulce* against maltase and sucrase enzymes was found to be 10.32±1.52 and 2.84±0.96 mg/ml respectively. Furthermore, the IC₅₀ values of methanolic extract of *P. dulce* against pancreatic α-amylase was found to be 16.75±1.81 mg/ml. The kinetics of glucosidase enzyme was determined by Lineweaver Burk plot and it was found to be non competitive in nature. Reversed phase HPLC analysis revealed oleanolic acid as the main triterpenoid constituent in the extract compared with standard oleanolic acid. Therefore, the enzyme inhibitory activity of *P. dulce* methanolic extract may be endorsed to the presence of oleanolic acid triterpenoid, thus justifying its traditional use in the management of diabetes.

Keywords: *Pithecellobium dulce*, Oleanolic acid, α-amylase, α-glucosidase.

Introduction

Diabetes is a chronic metabolic disorder in which homeostasis of the carbohydrate, protein and lipid metabolism is improperly regulated by the pancreatic hormone, insulin; resulting in an increased blood glucose level i.e. hyperglycemia. The hyperglycemia associated with the incidence and progression of microvascular (diabetic retinopathy, loss of vision and nephropathy) and macrovascular diseases (amputation and cardiovascular disease mortality) that are difficult to manage (Meenakshi et al., 2011; Prinya et al., 2012). The prevalence of diabetes is increasing annually and the number of diabetics is projected to rise above 300 million before 2025 (Ganiyu et al., 2012). Most prevalent form of diabetes is non-insulin dependent diabetes mellitus (NIDDM/type II) caused by impaired secretion of insulin and/or insulin action resulting in high postprandial glucose levels. One important factor to result in a postprandial hyperglycemia is the fast uptake of glucose in the intestine by the action of glucosidases, a class of enzymes (α-amylase and α-glucosidase) that helps in the breakdown of complex carbohydrates (starch and oligosaccharides) into simple sugars such as maltose and glucose (Hua-Qiang et al., 2012; Gray, 1995).

Therefore one of the important therapeutic approaches to decrease postprandial hyperglycemia is to retard absorption of glucose through inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase.

Currently a variety of therapeutic drugs are available for management of type 2 diabetes; these agents include hypoglycemic agents such as acarbose, miglitol and voglibose that competitively and reversibly inhibit α-glucosidase enzyme from intestine as well as pancreas. However, these drugs are associated with gastrointestinal side effects such as abdominal pain, flatulence and diarrhea in the patients, which might be caused by excessive inhibition of pancreatic α-amylase resulting in fermentation of undigested carbohydrates in the colon by colonic flora (Meenakshi et al., 2011; Suzuki et al., 2009). Therefore, a good strategy to managing postprandial hyperglycemia with lesser side effects is to identify the natural inhibitors from dietary sources, which has mild inhibitory effect against α-amylase and strong inhibitory activity against α-glucosidase (Kwon et al., 2006).

*Pithecellobium dulce* (Roxb.) Benth. (Manila Tamarind) belongs to the Mimosaceae family, mostly grown in India for hedges, street trees and for ornament because of its handsome foliage and curious pods. It is locally called as ‘Jungal jalebi’ and also known as ‘Vilayati babul’ in Hindi and ‘Vilayati chinch’ in Marathi. The seeds are stated to be eaten raw or in curries and seed oil is suitable for edible purposes and for soap manufacture (CSIR, 2003). Presence of steroids, saponins, triterpene oligoglycosides such as mixture of oleanolic acid and echiinocystic acid glycosides, lipids, phospholipids, glycosides, glycolipids and polyols has been
reported in the seeds (Nigam et al., 1997). A lysozyme has been isolated, purified and identified from P. dulce seeds with antifungal activity (Nuramon et al, 2011). The stem bark and leaves of P. dulce were reported to possess α-glucosidase inhibitory activity (Tanason et al., 2008). In our earlier study, we have reported antioxidant and free radical scavenging activity of extracts of P. dulce seeds (Dnyaneshwar et al., 2012). Traditionally the tender leaf paste is mixed with the seeds powder of P. dulce and is given orally in empty stomach to cure diabetes (Arul Manikandan et al., 2006). There is dearth of scientific reports on this plant properties based on anti diabetic activity despite its wide usage as medicinal plant. The present study, therefore investigated the α-amylase and α-glucosidase inhibitory potential of P. dulce seeds to corroborate antidiabetic activity.

Materials and Methods

Collection of Plant material

Seeds of P. dulce were collected during the month of August from Dhule District, Maharashtra, India. The plant material was authenticated by Dr. Ganesh Iyer, Botanist at Ruia College, Matunga, Mumbai. Freshly collected seeds were cleaned to remove adhering dust and then dried under shade. The dried samples were powdered in mixer grinder to a coarse powder and used for solvent extraction.

Extraction of Plant material

The air dried powdered seed material (100g) of P. dulce was successively extracted with pet ether (60-80°) and methanol using Soxhlet extractor. The methanol extract was concentrated by rotary evaporator and then dried (16 gm). The extract was stored in the refrigerator for subsequent analysis.

Chemicals, Kits and reagents

Porcine pancreatic α-amylase was procured from Sigma Aldrich Inc., (St Louis, MO). Dinitrosalicylic acid (DNSA) and Tris base was obtained from Himedia Laboratory, Mumbai. A glucose estimation kit was procured from Accurex Biomedical Pvt. Ltd., Thane, Mumbai. Starch, Maltose and Sucrose were purchased from Sisco Research Laboratories, (Mumbai, India). Acarbose was obtained from Bayer Medical Co. (Germany). All other chemicals and solvents used are of analytical grade.

Determination of total phenol content

The total phenolic content in the extract was determined with Folin-Ciocalteu reagent using the method of Sidduraju and Becker (2003). 100 µL of the extracts (10 mg/ml) were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm using a spectrophotometer (UV-1650, Shimadzu, Kyoto, Japan) against the reagent blank. The total phenolic content was calculated from a calibration curve using Gallic acid as a standard. The analysis was performed in triplicate and the results were expressed as the milligrams gallic acid equivalent/gram dry weight of extract.

Determination of flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination (Chang et al., 2002). Each plant extract (0.5 ml of 1:10 g/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm by using a spectrophotometer (UV-1650, Shimadzu, Kyoto, Japan). The flavonoid content was calculated from a calibration curve using Rutin as a standard. The analysis was performed in triplicate and the results were expressed as milligrams Rutin equivalent/gram dry weight extract.

Determination of total triterpenoids content

The total triterpenoids content was determined appropriately using the method described by Li et al., (2010). Briefly, the extract (250 µl) was mixed with the vanillin-glacial acetic acid (5% w/v, 0.25 ml) and 0.5 ml perchloric acid. The mixture was incubated at 60°C for 10 min, cooled in an ice bath for 15 min and then 2.5 ml glacial acetic acid was added and mixed well. After 6 min, the absorbance was read at 538 nm. Oleanolic acid was used as a reference standard and the content of the triterpenoids was expressed as Oleanolic acid equivalents (OAE, mg/g extract) through the calibration curve with oleanolic acid.

Preparation of rat intestinal α-glucosidase

Isolation of α-glucosidase from rat small intestine

The small intestine of male Wistar rats (180g) was collected after sacrificing the animal under anesthesia. The intestine was thoroughly cleaned with saline and epithelial layer (mucosal tissue) was collected by scraping the luminal surface firmly with a spatula. The mucosal scraping were homogenized in phosphate buffered saline (PBS) pH 7.4 containing 1 % triton X 100, and then centrifuged at 12000 rpm for 15 min. The supernatant fraction contained rat small intestinal α-glucosidase. Butanol was added to the supernatant fraction 1:1 proportion and centrifuged at 15000 rpm for 15 min. The aqueous layer was dialyzed overnight against the same buffer. After dialysis, the concentrated enzyme was used as crude α-glucosidase enzyme in the study to observe inhibition by extract of P. dulce seeds. All the preparations were carried out at 4 °C. The protein content of enzyme preparation was estimated by Lowry method (Lowry et al., 1951).

Effect of P. dulce on α-glucosidase inhibition assay

The effect of methanolic extract of P. dulce on rat intestinal α-glucosidase activity was assayed according to the method of Matsui et al., with slight modifications (Matsui et al., 2001). Briefly 0.5 mg protein equivalent of crude α-glucosidase enzyme was incubated with different concentrations of methanolic extract of P. dulce for 5 min before initiating the reaction with substrates maltose (6 mM) and sucrose (45 mM), in a final reaction mixture of 1 mL of 0.1 M phosphate buffer pH 7.2. The reaction mixture was incubated for 20 and 30 min at 37 °C for substrates maltose and sucrose, respectively. The reaction was stopped by adding 1.0 mL of Tris base and α-glucosidase activity was determined by monitoring the glucose released from maltose and sucrose by glucose oxidase method. Enzyme inhibition data were expressed as IC50 value (The concentration of P. dulce required to inhibit 50% of α-glucosidase activity).
Enzyme kinetic studies on inhibition of α-glucosidase enzyme by P. dulce

The enzyme kinetics on inhibition of α-glucosidase activity by methanolic extract of P. dulce was studied using different concentrations of substrate maltose (5, 10, 15, 20 and 25 mM) were incubated with α-glucosidase in the absence of inhibitor and with 7.5 and 15 mg/mL, for P. dulce in phosphate buffer pH 7.2 (0.1 M) at 37 °C, and amount of glucose formed was determined by glucose oxidase method. Double reciprocal plots of enzyme kinetics were constructed according to Lineweaver and Burk method to study the nature of inhibition. Kin and Vmax values were calculated from Lineweaver-Burk plots (1/S vs 1/v) (Lineweaver & Burk, 1934).

Inhibitory studies of P. dulce on α-amylase inhibition

α-Amylase activity was performed according to the chromogenic method described by Ali et al (2006). Briefly 120 µL of methanolic extract of P. dulce (20 mg/mL) in DMSO was mixed with 480 µL of distilled water and 1.2 mL of 0.5% w/v soluble potato starch in 20 mM phosphate buffer pH 6.9 containing 6.7 mM sodium chloride in a test tube. The reaction was initiated (0 min) by addition of 600 µL of enzyme solution (4 units/mL in distilled water), 600 µL of the mixture was withdrawn after 3 min into separate test tubes containing 300 µL DNSA color reagent (1 g of 3, 5-dinitrosalicylic acid (96 mM), 30g of sodium potassium tartrate and 20 mL of 2 N sodium hydroxide to a final volume of 100 mL in distilled water) and transferred to a hot water bath maintained at 85-90 °C for 15 min. Afterwards the reaction mixture in each tube was diluted with 2.7 mL distilled water and the absorbance measured at 540 nm (Shimadzu UV-160 spectrophotometer, Kyoto, Japan). Test incubations were also prepared for 5, 7.5, 10, 15 and 20 mg/mL of P. dulce to study the concentration dependant inhibition. For each concentration, blank incubations were prepared by replacing the enzyme solution with 600 µL in distilled water at the start of the reaction. Control incubations, representing 100% enzyme activity were conducted in a similar manner, replacing P. dulce with 120 µL DMSO. All the tests were run in triplicate. Net absorbance (A) due to the maltose generated was calculated as:

\[ A_{\text{sample}} - A_{\text{blank}} = A_{\text{test}} - A_{\text{blank}} \]

From the value obtained the percentage (w/v) of maltose generated was calculated from the equation obtained from the maltose standard calibration curve (0-0.1% w/v maltose). The level of inhibition (%) was calculated as:

\[ \% \text{ inhibition} = 100 \times \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{sample}} - A_{\text{blank}}}ight) \]

Where, % reaction = Mean maltose in sample × 100/ Mean maltose in control

Qualitative-quantitative high performance liquid chromatography (HPLC) analysis

The qualitative-quantitative analysis of methanolic extract of P. dulce was carried out using the method reported by Olszewska (2008). Methanolic extract (100 mg) is dissolved in methanol (2 ml), and then diluted with water (8 ml). Then 37% HCl solution (1 ml) was added to a portion of this solution (2 ml) and the mixture was refluxed for 3 hr, cooled and extracted with hexane (3 × 1 ml). The hexane extracts were pooled and evaporated to dryness and the residue was dissolved in methanol for HPLC-UV analysis. Agilent 1200 series chromatographic system (Agilent Technologies INC, USA) equipped with quaternary pump, degasser, auto sampler and variable wavelength detector was used for the analysis of extract.

Figure 1: The inhibitory effect of methanolic extract of P. dulce on A) intestinal glucosidase (maltase and sucrase) and B) porcine pancreatic α-amylase. The results are expressed as mean ± S.D., n=3.

The IC50 values calculated from dose response curves were found to be 10.32 ± 1.52 and 2.84 ± 0.96 mg/ml of P. dulce, respectively for substrates maltose and sucrase (Table 1). The inhibitory effect of P. dulce against sucrase was about 3 times higher than maltase hydrolysis activity.

Table 1. The IC50 values of methanolic extract of P. dulce against intestinal glucosidase (maltase and sucrase) and pancreatic α-amylase.

<table>
<thead>
<tr>
<th>Methanolic extract of P. dulce</th>
<th>IC50 values (mg/ml)</th>
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<tbody>
<tr>
<td>Intestinal sucrase</td>
<td>2.84 ± 0.96</td>
</tr>
<tr>
<td>Intestinal maltase</td>
<td>10.32 ± 1.52</td>
</tr>
<tr>
<td>Pancreatic α-amylase</td>
<td>16.75 ± 1.81</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± standard deviation of triplicate readings (n=3).

Kinetic study was carried out to understand the nature of inhibition of maltose hydrolysis by P. dulce seeds methanolic extract. Double reciprocal plot of inhibition of maltose hydrolysis is shown in Fig 2. The kinetic studies showed that P. dulce decreased the maximum velocity of enzyme activity or Vmax without much change in Km values (Table 2). The kinetic results demonstrated that the mechanism of α-glucosidase inhibition was of reversible, non-competitive nature.
The main constituent of methanolic extract of UV spectra comparison with standard oleanolic acid revealed that subjected to reverse phase HPLC analysis using retention time and oleanolic acid (0.244 mg/100 gm) (Fig. 3).

To characterize the bioactive compound responsible for inhibition of key carbohydrate hydrolyzing enzymes (intestinal α-glucosidase and pancreatic α-amylase), methanolic extract of P. dulce seeds was subjected to reverse phase HPLC analysis using retention time and UV spectra comparison with standard oleanolic acid revealed that the main constituent of methanolic extract of P. dulce seeds is oleanolic acid (0.244 mg/100 gm) (Fig. 3).

Table 2: Effect of methanolic extract of P. dulce (PDM) seeds on enzyme kinetics of rat intestinal α-glucosidase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Km (mM)</th>
<th>Vmax (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.3833</td>
<td>0.5556</td>
</tr>
<tr>
<td>PDM 7.5 mg/ml</td>
<td>6.3906</td>
<td>1.0173</td>
</tr>
<tr>
<td>PDM 15 mg/ml</td>
<td>6.4421</td>
<td>1.7544</td>
</tr>
</tbody>
</table>

Pancreatic α-amylase, another key carbohydrate hydrolyzing enzyme inhibitory activity of P. dulce was investigated. The effect of P. dulce on pancreatic α-amylase is presented in Fig. 1 B. The results revealed that the extracts inhibited α-amylase activity in a dose dependent manner (5-20 mg/ml), with IC50 value of (16.75 mg/ml).

P. dulce methanolic extract was subjected to quantitative phytochemical analysis of total phenolic, flavanoid and triterpenoids compounds analysis. The total phenolic, flavanoid and triterpenoids content of methanolic extract are presented in Table 3.

Table 3: Total phenolic, flavanoid and triterpenoids compounds in P. dulce seed extract

<table>
<thead>
<tr>
<th>Component</th>
<th>P. dulce Methanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Phenolic content</td>
<td>1.74 ± 0.0035 (mg/g Gallic acid equivalent)</td>
</tr>
<tr>
<td>Total Flavanoid Content</td>
<td>6.39 ± 0.122 (mg/g Rutin equivalent)</td>
</tr>
<tr>
<td>Total triterpenoids content</td>
<td>50.42 ± 0.532 (mg/g Oleanolic acid equivalent)</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± standard deviation of triplicate readings (n=3).

To characterize the bioactive compound responsible for inhibition of key carbohydrate hydrolyzing enzymes (intestinal α-glucosidase and pancreatic α-amylase), methanolic extract of P. dulce seeds was subjected to reverse phase HPLC analysis using retention time and UV spectra comparison with standard oleanolic acid revealed that the main constituent of methanolic extract of P. dulce seeds is oleanolic acid (0.244 mg/100 gm) (Fig. 3).

There are assorted number of plants and plant derived compounds have been used in treatment of diabetes to control blood sugar, as the synthetic antidiabetic drugs have adverse side effects in humans. Pancreatic and intestinal glucosidases are the key enzymes involved in hydrolysis of carbohydrates such as α-amylase and α-glucosidase, and inhibitors of these enzymes may be exploited as therapeutic approaches for controlling postprandial hyperglycemia (Shim et al, 2003; Meenakshi et al., 2011). Phytochemicals from plants such as phenolic compounds, saponins, flavonoids, glycosides, alkaloids etc. that are reported to play an important role in modulating glucosidase and amylase activities and therefore contribute to the management of postprandial hyperglycemia (Pulok et al., 2006; Ani and Akhilender, 2008). P. dulce seeds are commonly used as traditional herbs for medicinal properties such as anti-inflammatory, antifungal and antidiabetic. In this study, we report the inhibitory effect of saponin-rich extract of P. dulce seeds on rat intestinal α-glucosidase and pancreatic α-amylase activity and to characterize a bioactive compound responsible for the activity.

Discussion

Glucosidases located in the brush border surface membrane of intestine are key enzymes involved in production of glucose from the catabolism of oligosaccharides. Administration of glucosidase inhibitors are shown to control postprandial glucose levels by retarding digestion and absorption of carbohydrates (Ani and Akhilender, 2008). In the present study, we have shown that P. dulce inhibited both maltase and sucrase activities of intestinal epithelium in a dose dependent manner (1.25-20 mg/ml). The IC50 values for maltase and sucrase inhibition was found to be 10.32 ± 1.52 and 2.84 ± 0.96 mg/ml, respectively. The results revealed that the P. dulce has more sucrase inhibitory activity than maltase (Table 1). The kinetic results demonstrated that the nature of α-glucosidase inhibition was of non-competitive type; therefore P. dulce would bind to the enzyme at a region other than active site and may not be affected by higher concentration of substrate as against acarbose which is a competitive inhibitor with higher affinity toward sucrase than other disaccharidases.

Pancreatic α-amylase is involved in the breakdown of starch (polysaccharide) into disaccharides and oligosaccharides before intestinal α-glucosidase catalyzes the breakdown of disaccharides to release glucose which is later absorbed from small intestine into the blood circulation. Inhibition of these enzymes would slow down the breakdown of starch in the gastro-intestinal tract, thus retard...
digestion and absorption of carbohydrates, resulting in modulation of rise in postprandial hyperglycemia (Kwon et al., 2007). The results revealed that the methanolic extract of *P. dulce* inhibited α-amylase activity in a dose dependent manner (5-20 mg/ml), with IC₅₀ value of (16.75 mg/ml). This trend of the α-amylase inhibitory property of the methanolic extract agreed with their triterpenoids content (Table 3). Further, α-amylase inhibitory effect of mixture of oleanonic acid and ursolic acid (IC₅₀= 2.01 µg/ml) from the extract of *Phyllanthus amarus* support our results on inhibitory effect of triterpenes on α-amylase activity (Ali et al., 2006).

Pre incubation of oleanonic acid with rat intestinal α-glucosidase showed potent α-glucosidase inhibitory activity (IC₅₀ = 15 µM) (Ashok et al., 2010). Likewise, study has attributed the α-glucosidase inhibitory activity of medicinal plant foods to be a function of their triterpene content. Thus the presence of oleanonic acid might be responsible for the higher α-glucosidase inhibitory activity. However, the α-glucosidase inhibitory activities of *P. dulce* methanolic extract are higher than their α-amylase inhibitory activity (Table 1). These findings agreed with claims that plant phytochemicals are mild inhibitors of α-amylase and strong inhibitors of α-glucosidase (Kwon et al., 2007; Obob et al., 2010). The major drawbacks associated with synthetic α-glucosidase inhibitors due to their strong α-amylase and α-glucosidase inhibitory properties; resulting in excessive inhibition of pancreatic α-amylase leading to abnormal bacterial fermentation of undigested saccharides in the colon (Bischoff, 1994; Horii et al., 1987). Therefore, *P. dulce* has the potential to be used as a natural herbal drug capable of modulating carbohydrate hydrolyzing enzymes (α-glucosidase and α-amylase) and thus aid in the suppression of postprandial blood glucose levels.

Plant phytochemicals such as saponins like triterpenoids are known to cause insulin like effects and blocks the formation of glucose in the blood stream, which may be helpful in the treatment of diabetes (Bischoff, 1994; Horii et al., 1987). Polyphenolic components of black/bitter cumin *Centrtheratum anthelminticum* (L.) Kunze seeds. Eur Food Res Technol 226: 897-903


**References**


