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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 asses and characterizes 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, flavanoid and triterpenoids content by using Folin-Ciocalteu's reagent, Aluminum chloride ($AlCl_3$) 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 Dinitrosalicylic acid (DNSA) reagent, respectively. The IC_{50} 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 IC_{50} 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 echinocystic acid glycosides, lipids, phospholipids, glycosides, glycolipids and polysaccharides has been

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reported in the seeds (Nigam et al., 1997). A lysozyme has been isolated, purified and identified from *P. dulce* seeds with antifungal activity (Narumon et al, 2011). The stem bark and leaves of *P. dulce* were reported to possess α-glucosidase inhibitory activity (Tanasorn 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 antidiabetic 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 Sohxlet extractor. The methanol extract was concentrated by rotary vacuum 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 flavanoid 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 IC_{50} 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. K_m and V_{max} 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 tartarate 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_{540nm}*P. dulce* = A_{540nm}Test – A_{540nm}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:

% inhibition = 100-% reaction (at t=3 min)

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. The Hibar reversed phase C18 HPLC column (250 mm X 4.6mm, 5µm) was used as stationary phase. The mobile phase used was methanol and 1% (w/v) aqueous orthophosphoric acid in the proportion of 90:10. The flow rate was 0.6 ml/min, the injection volume was 20 µl and the detection was done at 206 nm and saponin constituent of the extract was identified using retention time and UV spectra comparison with standard oleanolic acid.

Data analysis

The data were expressed as mean ± standard deviation (SD) of results of three replicate readings (n=3). The IC_{50} values were calculated from plots inhibitor concentration versus percentage inhibition curves.

Results

In modern and traditional medicine, the plant derived phytoconstituents continue to provide valuable therapeutic agents to treat various ailments. In the present study, we have investigated the methanolic extract of *P. dulce* by testing its effect on some carbohydrate hydrolyzing enzymes *in vitro* and to characterize the bioactive compound responsible for the activity. Methanolic extract of *P. dulce* inhibited rat intestinal maltase and sucrase enzymes in a dose dependent manner as shown in Fig. 1 A.

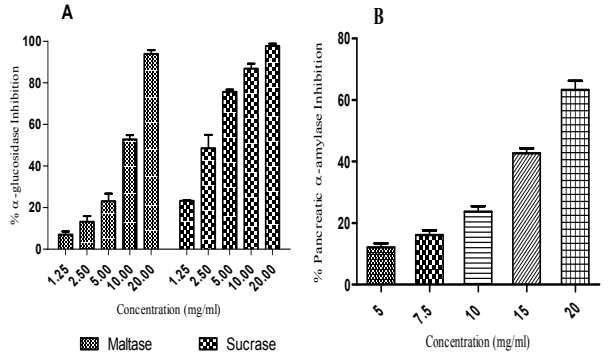

Figure 1: The inhibitory effect of methanolic extract of *P. dulce* on A) intestinal glucosidase (maltase and sucrase) and B) on porcine pancreatic α-amylase. The results are expressed as mean ± S.D., n=3.

The IC_{50} 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 sucrose (Table 1). The inhibitory effect of *P. dulce* against sucrase was about 3 times higher than maltase hydrolysis activity.

Table 1. The IC_{50} values of methanolic extract of *P. dulce* against intestinal glucosidase (maltase and sucrase) and pancreatic α-amylase.

Methanolic extract of *P. dulce*	**IC_{50} values (mg/ml)**
Intestinal sucrase	2.84 ± 0.96
Intestinal maltase	10.32 ± 1.52
Pancreatic α-amylase	16.75 ± 1.81

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 *V*max 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.

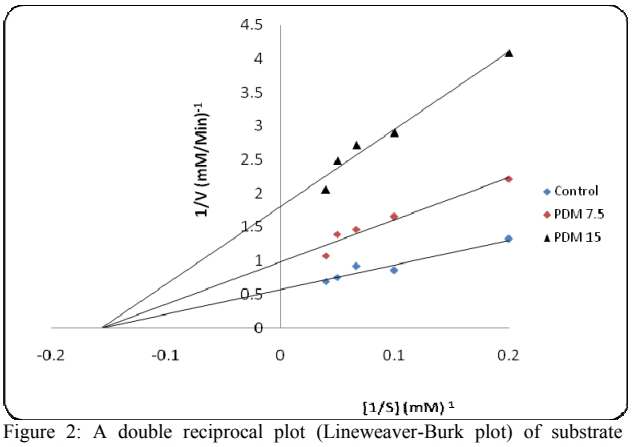

Figure 2: A double reciprocal plot (Lineweaver-Burk plot) of substrate dependent enzyme kinetics on inhibition of rat intestinal maltase activity by methanolic extract of *P. dulce* (PDM).

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

Treatment	*Km* (mM)	*Vmax*
Control	6.3833	0.5556
PDM 7.5 mg/ml	6.3906	1.0173
PDM 15 mg/ml	6.4421	1.7544

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 IC_{50} 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. The results of the study revealed that the triterpenoids content (50.42±0.532 mg oleanolic acid equivalent/g dried extract) of the extract was higher than the total phenolic (1.74±0.0035 mg Gallic acid equivalent/g dried extract) and flavanoid (6.39±0.122 mg Rutin equivalent/g dried extract) content. Earlier studies reported the presence of triterpene saponins of oleanolic acid and echinocystic acid glycosides in seeds of *P. dulce*, which is consistent with the results obtained in the present study.

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

Phytochemical Analysis	*P. dulce* Methanolic extract
Total Phenolic content (mg/g Gallic acid equivalent)	1.74 ± 0.0035
Total Flavanoid Content (mg/g Rutin equivalent)	6.39 ± 0.122
Total triterpenoids content (mg/g Oleanolic acid equivalent)	50. 42 ± 0.532

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).

Discussion

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,

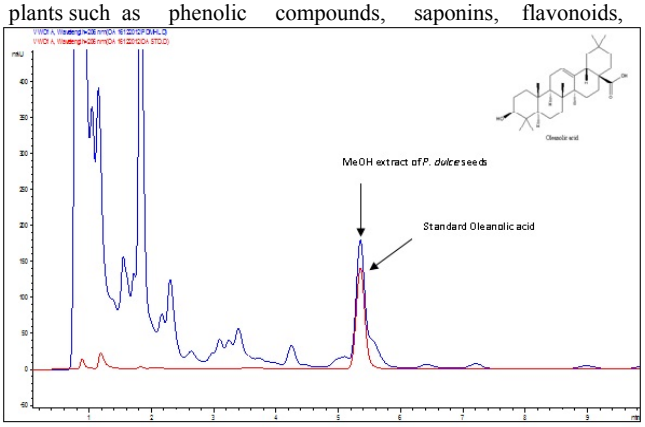

Figure 3: HPLC chromatogram of methanolic extract of *P. dulce* and standard oleanolic acid using the isocratic mobile phase at a flow rate of 0.6 ml/min

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.

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 IC_{50} 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 IC_{50} 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 oleanolic acid and ursolic acid (IC_{50}= 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 oleanolic acid with rat intestinal α-glucosidase showed potent α-glucosidase inhibitory activity (IC_{50}= 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 oleanolic 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; Oboh 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 (Pulok et al., 2006). The triterpenoids such as oleanolic acid, ursolic acid were reported to be good inhibitors of α-glucosidase and α-amylase. In our study, the methanolic extract of *P. dulce* seeds containing oleanolic acid showed significant inhibition of intestinal α-glucosidase (maltase and sucrase) and pancreatic α-amylase thus indicating a possible mechanism in antihyperglycemic effect of *P. dulce* seeds.

Conclusion

In conclusion, the present study demonstrated that *P. dulce* seeds are a good source of saponins with inhibitory activities against the carbohydrate hydrolyzing enzymes (intestinal α-glucosidase and pancreatic α-amylase). Inhibition of α-amylase and α-glucosidase activities by the extract may be attributed to their phenolic and triterpene constituent such as oleanolic acid. These results could be useful for developing functional food that modulating the key carbohydrate hydrolyzing enzymes such as intestinal α-glucosidase and pancreatic α-amylase and thus useful for management of diabetes mellitus particularly in control of postprandial hyperglycemia.

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