

Oleanolic acid - an α -Glucosidase inhibitory and antihyperglycemic active compound from the fruits of *Sonneratia caseolaris*

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Abstract: Fruits of *Sonneratia caseolaris* Linn. have many therapeutic applications in folklore medicine. However, chemical examination and biological activity of its fruit are less studied. During the work on identification of antidiabetic principles from Indian mangrove flora, we noticed moderate intestinal α -glucosidase inhibitory activity of the methanolic extract of its fruits. Three compounds namely oleanolic acid, β -sistosterol- β -D-glucopyranoside and luteolin were isolated and identified from the bioactive methanolic extract. *In vitro* pre-incubation of crude rat intestinal α -glucosidase with oleanolic acid showed potent α -glucosidase inhibitory ($IC_{50} = 15 \mu\text{M}$) activity, however, its inhibitory potential decreased drastically when oleanolic acid was pre-incubated with substrate. In *in vivo* studies also, pretreatment of rats with oleanolic acid displayed significant ($p < 0.05$) antihyperglycemic activity in starch tolerance test however, administration of starch fortified with oleanolic acid to the rats could not exhibit antihyperglycemic activity. This is the first report identifying oleanolic acid in *S. caseolaris* fruits in substantial yield and assigning its intestinal α -glucosidase inhibitory and antihyperglycemic activities. This study also highlights the fact that the mode of incubation in *in vitro* experiments and administration of active principles isolated from natural medicinal plants in *in vivo* studies play important role in revealing various facets of their biological activities and therapeutic implications.

Key words: Antidiabetic activity, crabapple, mangroves, oleanolic acid.

Introduction

Mangroves provide food, variety of traditional products, artefacts and are used in folk medicine. However, knowledge about their biological activities and chemical constituents is still in its infancy. During the work on identification of antidiabetic principles from Indian mangrove flora (Mangala Gowri *et al.*, 2007), we noticed intestinal α -glucosidase inhibitory property in methanolic extract of the fresh fruits of *Sonneratia caseolaris* (Family: Sonneratiaceae).

S. caseolaris is a well-known mangrove tree, native of South and South East Asia. It is commonly located in mangrove forests of silty tropical shores of Sri Lanka to Malay Peninsula and northern Australia. The fruit is commonly known as Ora or Crabapple. The immature berries are sour in taste and edible, used as fruit vegetable and flavoring agent. However, mature fruits have a cheese like taste and are eaten raw or cooked. In folk medicine, fruit is used as remedy to stop bleeding and in the treatment of piles and cough, removal of intestinal worms and as sprain poultices (Bandaranayake, 2002). It has also been found toxic to mosquito larvae (Devi *et al.*, 1997) and possess hepatoprotective activity (Charoenteeraboon *et al.*, 2007). Fatty acids, sterols,

hydrocarbons (Hogg & Gillan, 1984), flavonoid, luteolin and its glycosides (Sadhu *et al.*, 2006) have been reported from its leaves but no chemical examination and biological activity of its fruits have been reported.

In this study, chemical examination of the methanolic extract of the fresh fruit of *S. caseolaris* and intestinal α -glucosidase inhibitory and antihyperglycemic active principle have been reported for the first time.

Materials and Methods

Plant material

Fresh fruits of *S. caseolaris* were collected from Ernakulam coast (Kerala State) of India and were authenticated by Dr. Venkaiah, Department of Botany, Andhra University, Vishakapatnam and a voucher specimen (No. IIC-MG-103) was preserved in the Organic Division-1, Indian Institute of Chemical Technology, Hyderabad, India.

General experimental procedures

Thin layer chromatography (TLC) was performed on precoated silica gel GF₂₅₄ plates (Merck) using ethyl acetate:hexane (40:60) as a developing solvent and plates were visualised under UV light or by spraying with 10% methanolic sulphuric acid and

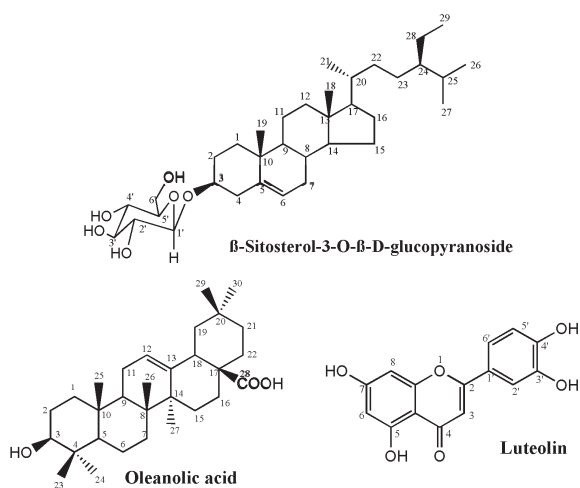


Fig. 1. Structures of compounds isolated from the fruits of *S. caseolaris*

heated at 110°C till appearance of spots. The ^1H NMR and the ^{13}C NMR spectra were recorded on Varian Gemini 200MHz, Bruker Avance 300 MHz and Bruker Avance 75MHz, respectively. The IR spectra were recorded using a Perkin Elmer FT-IR and ESI-MS on LC-MSD-Trap-SL ion trap detector (Agilent-1100 series). The optical rotation was determined using a Horiba SFPA-300.

Extraction and isolation

Fresh fruits (5 kg) were extracted with methanol (5 L \times 3 times) at ambient temperature to yield crude methanol extract (8 g). The methanol extract (6 g) was subjected to vacuum liquid chromatography over a column of silica gel (230-400 mesh) eluted with a gradient of hexane and ethyl acetate of increasing polarity to give three fractions. Further purification of these fractions over a column of silica gel furnished three compounds when eluted with hexane:ethyl acetate (8:2, 6:4) and ethyl acetate:methanol (9.5:0.5) mixtures. These compounds were identified as oleanolic acid, luteolin and β -sitosterol- β -D-glucopyranoside (Fig. 1) respectively, by direct comparison of their co-TLC, IR, ^1H NMR and mass data with authentic samples.

In vitro α -Glucosidase inhibitory assay

Intestinal α -glucosidase inhibitory activity was determined as reported earlier (Mangala Gowri *et al.*, 2007). Rat intestinal acetone powder in normal saline (100:1; w/v) was sonicated properly and the supernatant was used as a source of crude intestinal α -glucosidase. In brief, 10 μL of test samples [5 mg mL^{-1} dimethyl sulphoxide (DMSO) solution] were reconstituted in 100 μL of 100 mM-phosphate buffer (pH 6.8) in 96 well microplate and incubated at 26°C with 50 μL of crude intestinal α -glucosidase for 5 minutes before 50 μL substrate [5 mM, *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG) prepared in same

buffer] was added. Release of *p*-nitrophenol was measured at 405 nm spectrophotometrically (Spectra_{MAX} Plus³⁸⁴, Molecular Devices Corporation, USA) 5 minutes after incubation with substrate. Individual blanks for test samples were prepared to correct background absorbance where substrate was replaced with 50 μL of buffer. Control sample contained 10 μL DMSO in place of test samples. Percentage of enzyme inhibition was calculated as $(1 - B/A) \times 100$ where, A represents absorbance of control without test samples and B represents absorbance in presence of test samples. At least five serial dilutions of the primary test samples (5 mg mL^{-1} DMSO) were considered to study concentration dependant enzyme inhibition assay and calculation of IC_{50} values (concentration required to inhibit 50% enzyme activity). All the tests were run in triplicate. The IC_{50} value was calculated applying logarithmic regression analysis from the mean inhibitory values at individual concentrations.

Starch tolerance test

Antihyperglycemic activity was carried out according to the method reported earlier (Tiwari *et al.*, 2008). Male Wistar rats weighing between 195 g and 215 g were obtained from National Institute of Nutrition (CPCSEA Reg. No. 154, Government of India), Hyderabad. The animals were housed in standard polyvinyl cages and room temperature was maintained at 22 ± 1 °C with an alternating 12 h light and dark cycle. Food and water were provided *ad libitum*. Experiments were performed as per the Institutional Animal Ethical Committee norms. The rats were divided into two groups *viz.* control and oleanolic acid (OA) treated, containing six rats in each group. All the animals were kept for overnight fasting. Next day forenoon blood was collected from retro orbital plexus in EDTA containing tubes and plasma glucose levels for basal (0-h) value were measured by glucose-oxidase method using Auto blood analyzer instrument (Bayer EXPRESS PLUS). Oleanolic acid was suspended in normal saline and administered orally in the dose of 50 mg/kg body weights. The control group of animals was given only normal saline. Fifteen minutes after test sample treatment, animals were fed with soluble-starch dissolved in normal saline at a dose of 2 g/kg body weight. Thereafter, blood was collected at intervals of 30, 60, 90 and 120th minutes post starch feeding. Plasma was separated and glucose level was measured as described above.

In another set of animal experiment, test group of rats were given starch fortified with 2.5% oleanolic acid, while control group received only starch solution. Plasma glucose levels were analyzed accordingly.

Results and Discussion

Excessive intake of high calorie and quickly digestible high carbohydrate diet results in abnormal surges in blood glucose level, also called postprandial hyperglycemic (PPHG) excursion (Wolver & Mehling, 2003; O'Keefe & Bell, 2007). PPHG has also been found to be responsible of inducing oxidative stress (Ceriello & Motz, 2004) and development diabetic complication (Wright *et al.*, 2006) Therefore, modulation of dietary carbohydrate digestion has become an effective tool to attenuate PPHG excursion. Inhibitors of intestinal brush border enzyme, α -glucosidases have been observed to slow down the rate of carbohydrate digestion including starch and disaccharides (O' Keefe & Bell, 2007). α -glucosidase inhibitory drug, acarbose, acts specifically at the level of postprandial hyperglycemia. However, the side effects such as abdominal distention, flatulence and possibly diarrhoea caused by excessive inhibition of pancreatic α -amylase by acarbose, which results in abnormal bacterial fermentation of undigested carbohydrate in the colon limits its use (Baily, 2003). Therefore, α -glucosidase inhibitor from natural resources could offer better therapeutic strategy to control PPHG excursion and provide benefit without undesirable effects.

Methanolic extract of the fresh fruits of *S. caseolaris* exhibited intestinal α -glucosidase inhibitory activity (Fig. 2) in rats. Oleanolic acid, a major compound present in the methanolic extract showed strong enzyme inhibitory activity ($IC_{50} = 15 \mu\text{M}$; Fig. 3), however, two other minor compounds namely β -sistosterol- β -D-glucopyranoside and luteolin were less active inhibitors. Nevertheless, it is not always necessary that *in vitro* inhibitory activity may relate

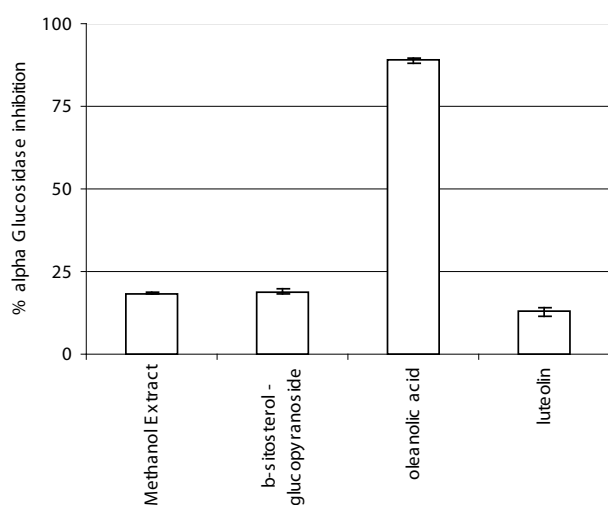


Fig. 2. Rat intestinal α -glucosidase inhibitory activity of extract and compounds isolated from *Sonneratia caseolaris* (at 50 $\mu\text{g}/\text{mL}$ concentration). Data represents mean \pm SD of triplicate samples

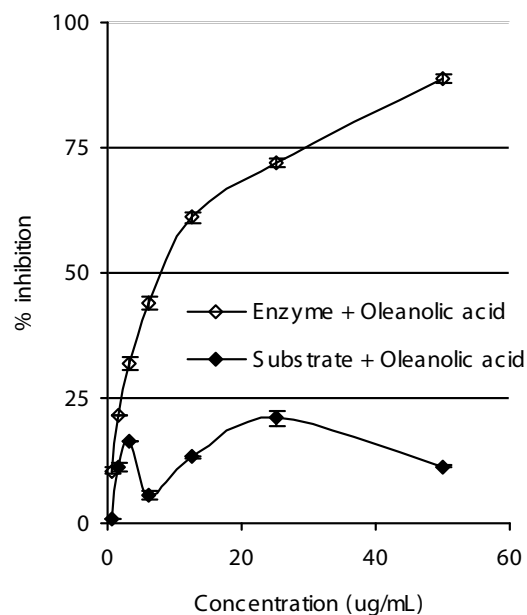


Fig. 3. *In vitro* intestinal α -Glucosidase inhibition assay. $[\diamond]$ represents percentage of enzyme inhibition where test compound oleanolic acid was incubated first with the enzyme and then substrate *p*-NPG was added to the reaction mixture however, $[\blacklozenge]$ presents enzyme inhibition pattern from the experiments where oleanolic acid was first incubated with the substrate *p*-NPG and then enzyme was added to the reaction mixture. Values represent mean \pm SD of triplicate samples.

to the corresponding *in vivo* activity (Subramanian *et al.*, 2008). Therefore, in order to check for the proof of concept, oleanolic was selected for evaluation of its potency in mitigating PPHG excursion following starch tolerance test in Wistar rats. Oleanolic acid was administered to rats fifteen minutes before starch tolerance test showed considerable reduction in PPHG excursion up to first 60 minutes (Fig. 4 A).

In this experiment, pretreatment of rats with oleanolic acid resulted in 22 % ($p < 0.05$), 21 % ($p < 0.05$) and 12 % ($p < 0.05$) less increase in plasma glucose level after 30, 60 and 90 minutes, respectively of starch feeding in comparison to the control group of rats (Fig. 4 A). Matsuda *et al.* (1998) reported that pretreatment of rats with oleanolic acid could not reduce serum glucose level in glucose tolerance test even with 100 mg/kg body weight dose of oleanolic acid at 30 minute of oral glucose load. This may be due to the reason that oleanolic acid is a α -glucosidase inhibitor hence suitable for *in vivo* starch tolerance test. Therefore, in oral glucose tolerance test it may not display antihyperglycemic activity like other natural α -glucosidase inhibitors (Subramanian *et al.*, 2008). Our observation provides evidence for the first time that oleanolic acid reduces starch induced PPHG excursion through inhibition of intestinal

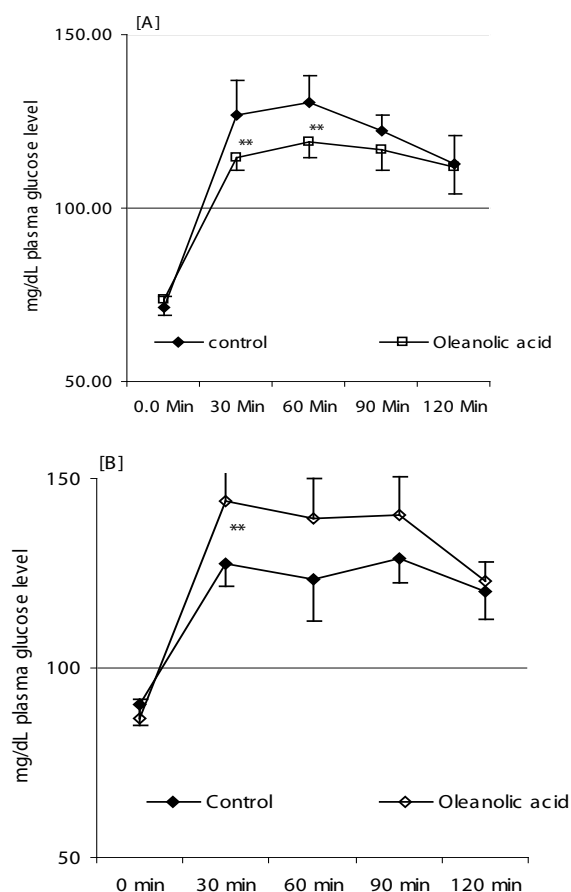


Fig. 4. Starch tolerance tests. [A] Rats were given orally, oleanolic acid (50-mg/kg body weights), 15 minutes before administration of soluble starch (2 gm/kg body weight). [B] Soluble starch (2 gm/kg body weight) was fortified with oleanolic acid (50-mg/kg body weights) for oral administration to the rats. In both experiments, the control groups of animals were treated with vehicle in which test compounds were suspended. One-way ANOVA was used to study the changes produced after starch feeding using Newman-Keuls Multiple Comparison Test to compare differences between the groups. ** $P < 0.05$ when compared with control. Values represent mean \pm SD of six animals in experiment [A] and five in experiment [B].

α -glucosidase enzyme.

Oleanolic acid is well known to possess anti-inflammatory, antihyperlipidemic and hepatoprotective activities and sold as oral drug for human liver disorder in China (Liu, 1995). It has been warranted that in low dose, oleanolic acid is hepatoprotective, produces adaptive responses, while the high doses, could produce cholestasis and hepatotoxicity (Liu, 2005). Oleanolic acid, a ubiquitous triterpenoid is also an integral part of the human diet. Therefore, to test the hypothesis that

whether presence of oleanolic acid in diet has any glycemic importance, we incubated oleanolic acid *in vitro* with substrate *p*-NPG for the first five minutes and then added crude α -glucosidase enzyme in the reaction mixture. It was observed that oleanolic acid could not display potent α -glucosidase inhibition (Fig. 3). Furthermore, when rats were orally administered starch fortified with oleanolic acid, it could not produce any antihyperglycemic activity. Rather blood glucose level in this group of animals was found increased than the group of control animals, who received only starch (Fig. 4B). In our earlier *in vitro* study (Tiwari *et al.*, 2007) also the drastic difference in potency for intestinal α -glucosidase inhibitory activity for spices extracts was observed under similar incubation conditions. Majority of non-glycoside α -glucosidase inhibitors identified in natural products have been reported as non-competitive inhibitor of α -glucosidase (Matsui *et al.*, 2006). The IC_{50} value of a substrate-specific-competitive intestinal α -glucosidase inhibitor drug, acarbose was found to be 13 μ M when pre-incubated with the enzyme and 37 μ M when reacted in the presence of substrate (Tiwari *et al.*, 2007). Therefore, possible difference in the potency for activity of oleanolic acid under different experimental conditions may not be surprising due to the apparent reduction in inhibitory activity on the enzyme through substrate-competition and affinity of the enzyme towards inhibitor or the substrate (Nasu *et al.*, 2005).

It is important to note that although the research with pure natural compounds delineates mechanism of their biological actions and help in development of new therapeutics, in traditional medicines they are present in mixtures and that mixture of compounds in traditional medicinal preparations are thought to be more effective than a single compound (Liu, 2005). However, in the light of the observations made in our study, the health effects of a compound present in a diet or a traditional medicinal formulation and its therapeutic property needs to be explained with caution. Because our study reveals that presence of oleanolic acid in diet may be responsible for adding nutritional value in the diet like better digestion of carbohydrates however, in pure form it may have therapeutic implications in control of diet induced hyperglycemia.

Acknowledgements

Authors are thankful to the Dr. J. S. Yadav, Director, IICT, Hyderabad for his constant encouragement.

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Manuscript received: 13 April, 2009

Manuscript accepted: 25 February, 2010