

Identification of α -Glucosidase Inhibitors From *Psidium guajava* Leaves and *Syzygium cumini* Linn. Seeds

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Abstract

We screened for α -glucosidase inhibitors from 12 traditional medicinal plants used for the treatment of diabetes. Potent α -glucosidase inhibitory activity was found in aqueous extract of *Psidium guajava* leaves and *Syzygium cumini* Linn seed extract. Active principles inhibiting α -glucosidase were isolated and identified as apigenin 7-O-glucoside in the *S.cumini* Linn seed extract and Dihydro-3,3',4',5,7 – pentahydroxyflavone glycoside in *P.guajava* leaf extract through FT-IR and LC-MS. The inhibitor present in both *P.guajava* leaves and *S.cumini* Linn seed extract was found to be reversible in nature.

Keywords: Diabetes, α -glucosidase, Medicinal plants, *P.guajava*, Reversible inhibitor, *S.cumini*

1. Introduction

Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (Andrade-Cetto *et al.*, 2008). Enzyme inhibitors have potential value in many areas of disease control and treatment. The control of kinetics of carbohydrate digestion and monosaccharide absorption could be of value in the prevention and control of conditions such as diabetes, obesity, hyperlipoproteinaemia and hyperlipidaemia, and in these respect inhibitors of glucosidase are of particular interest (Kim and Nho, 2004). Glucosidases (EC 3.2.1.20) are located in the intestinal brush-border surface of small intestinal cells and they catalyze the cleavage of glycosidic bonds in oligosaccharides or glycoconjugates. Therefore, an inhibitor of intestinal α -glucosidase could be expected to retard carbohydrate digestion and absorption (Ohta *et al.*, 2002; Borges de Melo *et al.*, 2006). Potent α -glucosidase inhibitor, acarbose, a tetrasaccharide synthesized by *Actinomyces* has been reported to blunt postprandial rise in plasma glucose. However, its use has been limited by the side effects such as flatulence and diarrhoea. The α -glucosidase inhibitors with molecular weights below 250, which can be absorbed appreciably from the gut into the bloodstream, are arousing great interest as therapeutic agents (Kim and Nho, 2004). Therefore, present study was targeted towards the identification of low molecular weight α -glucosidase inhibitor from medicinal plants and the hypoglycemic activity of the selected twelve medicinal plant were evaluated with respect to their ability to inhibit intestinal α -glucosidase.

2. Materials and Methods

2.1 Plant materials

Medicinal plants *Psidium guajava*, *Syzygium cumini*, *Butea frondosa*, *Nelumbo nucifera*, *Catharanthus roseus*, *Cuminum cyminum*, *Andrographis lineate*, *Abrus precatorius*, *Costus speciosus*, *Punica granatum*, *Artemisia pallens* and *Ocimum sanctum* (Subramoniam *et al.*, 1996; Li *et al.*, 2004; Bnouham *et al.*, 2006; Rai *et al.*, 2007; Ayyanar *et al.*, 2008; Deore *et al.*, 2008) were selected for the investigation because of their previously reported hypoglycemic effects and their usage in the treatment of diabetes. The different medicinal plant parts used for the present investigation were collected from botanical garden, Tamil Nadu Agricultural University (TNAU), Coimbatore, India and some were purchased from the medicinal herb market in Coimbatore city and all of them were botanically identified and authenticated. Voucher specimen of the plant materials were deposited at TNAU herbarium viz TNAU 1459 to TNAU 1470 respectively as in the order described above.

2.2 Preparation of plant extract

Five gram of the desired fresh plant parts were taken and washed well with distilled water and ground with 30ml of distilled water using ice cold mortar and pestle. Then the homogenates were centrifuged (Kubota 3700 model) at 10,000rpm for 20min and the supernatant were used as crude inhibitor source.

2.3 Preparation of crude α -glucosidase from rat small intestine

Rat small intestine homogenate was prepared according to the method described by Lossow et al.,(1964) with slight modifications. Rats were exsanguinated by withdrawal of blood from the heart (usually 8-10 ml) under chloroform anesthesia. Small intestine was removed and washed with 30ml of 0.9% NaCl and placed in ice cold 0.9% NaCl. The intestine was minced with a surgical knife and homogenized using Potter-Elvehjem type of homogenizer in 50 ml of 0.1 M potassium phosphate buffer of pH 6.8. After 30 min, the homogenates were centrifuged for 30 min at 10,000 rpm at 4°C. The Supernatant was used as crude enzyme source.

2.4 α - Glucosidase inhibitor assay

The α -glucosidase inhibitory activity was determined by measuring the release of 4-nitrophenol from P-nitrophenyl α -D glucopyranoside. The assay mixtures for these experiments contained 0.3 ml of 10mM p-nitrophenyl α -glucopyranoside, 1.0ml of potassium phosphate (0.1M, pH: 6.8), 0.2 ml of crude enzyme solution and 0.2 ml of inhibitor extract, all in a final volume of 1.7 ml. Following an incubation time of 30 min at 37°C, the reaction was terminated by the addition of 2.0 ml of 100 mM sodium carbonate. The liberated p-nitrophenol was determined at 400nm using spectrophotometer (Beckman coulter DU 530 model). The % inhibition rates were calculated using the formula, % inhibition= $100 \times (\text{Absorption}_{\text{control}} - \text{Absorption}_{\text{test}}) / \text{Absorption}_{\text{control}}$.

2.5 Ammonium sulphate precipitation

Ammonium sulphate precipitation (0-100% F) was carried out as described by Sadasivam and Manickam (2004). To 10ml of the extract, 7.07 gram of ammonium sulphate was added under constant stirring conditions, and then the contents were centrifuged at 6000rpm for 20 min. The supernatant and pellet were further checked for their inhibitory activity.

2.6 Phenol estimation

Total phenol content of the extract was determined according to the Folin-Ciocalteu colorimetric method as described by Maher *et al* (2006). Briefly, to 50 μ l of each sample, 500 μ l of the Folin-Ciocalteu reagent and 2ml of 10% sodium carbonate were added before incubating at room temperature for 3 min. The absorbance of each sample was measured spectrophotometrically at 650nm using spectrophotometer (Beckman coulter DU 530 model).

2.7 Separation and purification of compound

The aqueous extract was subjected to thin layer chromatography (using silica gel, G) to identify the chemical nature of the inhibitor compound. Different solvent systems were employed and best separation was observed with ethyl acetate: ethanol: water = 5:1:5. The plates were then developed by spraying 1% ethanolic solution of aluminium chloride and Dragendroff reagent to identify the presence of flavonoids and alkaloids respectively.

The fraction containing α -glucosidase inhibitory compounds were subjected to silica gel column chromatography. The active components were separated successively using the following solvent system: ethanol, ethanol:water (1:1 v/v) and water. The fractions collected were assayed for their flavonoid content & pooled together and checked for their inhibitory activity.

Flavonoid content was determined using aluminium chloride colorimetric method. Briefly, to 0.1ml of the sample, 75 μ l of 5% sodium nitrite was added and incubated at room temperature for 5min. Then, 150 μ l of 10% aluminium chloride were added and incubated at room temperature for 6min. The absorbance of each sample was measured spectrophotometrically at 510nm using spectrophotometer (Beckman coulter DU 530 model) after the addition of 0.5ml of 1M sodium hydroxide (Sathishkumar *et al.*, 2008).

2.8 HPLC

The active fraction showing maximum inhibition towards α -glucosidase was checked for its purity by RP-HPLC using Shimadzu HPLC model RP4A, C18 5 μ column (4.6 X 250mm) equipped with variable UV detector. The compound were eluted at the flow rate of 1ml/min using acetonitrile (A) and water containing 0.1% phosphoric acid (B) using the gradient program (0-5min- 5%A, 95%B; 6-10min- 10%A, 90%B; 11-25min- 15%A,85%B; 26-45min- 40%A, 60%B; 46-55min- 95%A, 5%B; 56-65min- 5%A, 95%B). Wavelength was set between 200-400nm, retention at 285 and 365nm.

2.9 Spectral analysis

The purified fractions which showed maximum inhibition for α -glucosidase, was further analyzed through FT-IR available at Indian Institute of Technology (IIT), Chennai, India and LC-MS available at Regional Sophisticated instrumentation Center, Central Drug Research Institute (CDRI), Lucknow, India.

2.10 Dialysis

The solution containing α -glucosidase (0.2ml), crude inhibitor extract (0.2ml) and potassium phosphate buffer (1ml) were incubated for 2hr at room temperature and then were dialyzed against potassium phosphate buffer (0.1M, pH: 6.8) at 4^oC for 12h. The buffer was changed at every 6hr. The aliquot of 0.5ml kept at 4^oC for 12hr without dialysis served as control.

3. Results and discussion

3.1 α - Glucosidase inhibitory fractions and their phenolic content

Pancreatic and intestinal glucosidases are the key enzymes of dietary carbohydrate digestion and inhibitors of these enzymes may be effective in retarding glucose absorption (Bhat *et al.*, 2008). This is because only monosaccharides are readily taken up from the intestine and all other carbohydrates have to be broken-down enzymatically before they can be absorbed. Out of twelve medicinal plants screened for α -glucosidase inhibitor, the aqueous extract of *Psidium guajava* showed strong inhibition (99.5%) followed by *Abrus precatorius* (76.8%) whereas *Butea frondosa* (flower) showed least inhibition 6.22%. Among the plant extracts which showed inhibition against α -glucosidase, the total phenolic content were high in *P. guajava* leaves and *Syzygium cumini* seed. IC₅₀ values were determined using dose response curves for the plant extracts which showed inhibitory activity and their total phenolic content were determined and are shown in Table 1.

Table 1 - Inhibition of α -glucosidase by various plant extracts from medicinal plants and their total phenol content along with their IC50 values

Scientific name	Plant part	% inhibition	Concentration of phenol (mg/g of fresh plant material)	IC50 values (mg/mL) (mean \pm SD)
<i>Psidium guajava</i>	Leaf	99.50	250	10 \pm 0.04
<i>Syzygium cumini</i>	Seed	75.72	180	8 \pm 0.17
<i>Andrographis lineate</i>	Leaf	No inhibition	-	-
<i>Abrus precatorius</i>	Leaf	76.81	61	212 \pm 0.18
<i>Costus speciosus</i>	Rhizome	No inhibition	-	-
<i>Nelumbo nucifera</i>	Petal	13.95	48	868 \pm 0.23
	Stem	No inhibition	-	-
<i>Catharanthus roseus</i>	Leaf	9.48	56	80 \pm 0.25
<i>Punica granatum</i>	Peel	No inhibition	-	-
<i>Butea frondosa</i>	Leaf	47.92	58	35 \pm 0.42
	Flower	6.22	23	118 \pm 0.17
<i>Artemisia pallens</i>	Leaf	53.68	26	29 \pm 0.35
<i>Ocimum sanctum</i>	Leaf	40.24	62	38 \pm 0.42
<i>Cuminum cyminum</i>	Seed	71.52	53	24 \pm 0.13

Phenolic fractions of plants have long been recognized to inhibit carbohydrate hydrolyzing enzymes in mammals. Phenolic compounds derived from red cabbage, strawberries and raspberries are also inhibitors of α -amylase and α -glucosidase (Mc Dougall *et al.*, 2005). Results of the present study suggest that phenolic compounds of *P. guajava* leaves and *S. cumini* Linn seed can play a role in managing diabetes via the inhibition of α -glucosidase. It is not clear yet which kind of mechanism results in the inhibition of α -glucosidase. Certainly, high concentrations of any plant extract will always inhibit enzymes by non-specific inhibition or denaturing of the enzyme (Schäfer and Högger, 2007). However, in the case of *P. guajava* leaves (IC₅₀=10 mg/ml) and *S. cumini* Linn seed (IC₅₀=8 mg/ml) extract, the concentration was rather low. Thus it can be assumed that the inhibition of α -glucosidase by these extracts were not solely due to non-specific binding or denaturing of the enzyme protein. Interestingly, the phenolic content of *B. frondosa* (Flower), *Nelumbo nucifera* (Petal), and *Artemisia pallens* extract is less than 50 mg/g of fresh plant material, indicating that inhibitory activity of these extracts may be due to the presence of other inhibitor compound(s).

3.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation (0-100% F) was carried out for all the plant extracts which showed inhibition against α -glucosidase. The nature of inhibitor was found to be non-Proteinaceous in case of *P. guajava*, *S. cumini*, *B. frondosa*, *N. nucifera*, *C. roseus* and *C. cyminum* whereas in the case of other extracts it was found to be proteinaceous in nature.

Out of twelve medicinal plant extract screened, *P. guajava* and *S. cumini* were selected for further study as the present study was aimed at the identification of potent, low molecular weight non-proteinaceous inhibitor. The type of inhibition exhibited by *P. guajava* and *S. cumini* extract was found to be competitive and non-competitive respectively as characterized using Dixon plot. Aqueous extract of *P. guajava* and *S. cumini* pre-incubated with α -glucosidase was dialyzed against buffer and it was found that the % inhibition of the samples without dialysis (control) was greater than the dialyzed samples indicating that the mode of inhibition is reversible (Data not shown).

3.3 Separation and purification of compound

Preparative thin layer chromatography was performed to separate the active component and the plates were developed by spraying 1% ethanolic solution of aluminium chloride to identify the presence of flavonoids.

The fraction with R_f value 0.88 to 0.92 (*P. guajava*) & 0.86 to 1.00 (*S. cumini*) in preparative TLC, which showed maximum inhibition towards α -glucosidase were further separated by silica gel column chromatography. The collected fractions were pooled together based on their flavonoid content and checked for their purity by RP-HPLC.

3.4 Spectral analysis

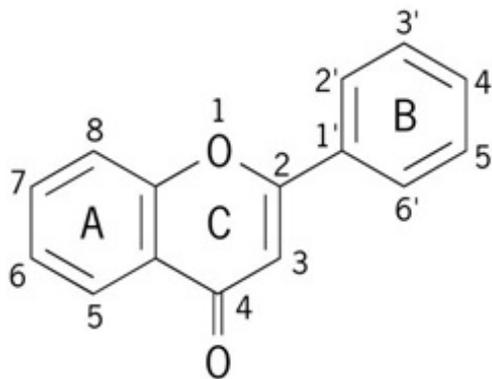
The Mass spectral analysis of the purified sample obtained from *P. guajava* leaves revealed the presence of Dihydro- 3, 3', 4', 5, 7 – pentahydroxyflavone glycoside (FT-IR: 1419 and 1382 – phenol OH, 1654 – unsaturation due to C=C; 1275- Phenol –CO stretch) & *S. cumini* Linn seed purified aqueous extract revealed the presence of apigenin 7-O-glucoside (FT-IR: 1332- Phenol; 1276- Phenol –CO stretch). The molecular ion peak of Dihydro- 3, 3', 4', 5, 7 – pentahydroxyflavone glycoside was found at m/z 303, and apigenin 7-O-glucoside at m/z 433.

S. cumini seed powder has been reported to lower glucose level in various animal models (Sridhar *et al.*, 2005; Bnouham *et al.*, 2006). The acetone extract of *S. cumini* seed was found to be a potent inhibitor of α -glucosidase hydrolysis of maltose in *in-vitro* and *in-vivo* Gato-Kakazaki (GK) rats (Shinde *et al.*, 2008). The aqueous extract of *P. guajava* leaves has a good effect to lower blood glucose and various parts have been used for the treatment of Diabetes mellitus (Li *et al.*, 2004; Rai *et al.*, 2007). Maruyama *et al.*, (1985) have already reported the possible hypoglycemic activity of flavonoid glycosides present in *P. guajava* leaf extract.

The poly phenols in plants have been reported to play an important role in the regulation of carbohydrates hydrolyzing enzymes (Mai & Chuyen., 2007). Glycosides, flavanoids, tannins and alkaloids have been reported to possess hypoglycemic activities (Kumar *et al.*, 2009). In the present investigation, flavanoid glycoside isolated from *P. guajava* and *S. cumini* was found to inhibit α -glucosidase which is in accordance with the findings of Jung *et al.*, (2006) who have shown inhibition of α -glucosidase by flavanoid glycoside isolated from five different plants.

Ishikawa *et al.*, (2007) studied the inhibition of α -glucosidase by various poly phenolic compounds. Of the compounds tested, quercetin, catechin and epicatechin were reported to inhibit α -glucosidase strongly when compared to apigenin indicating that presence of two hydroxyl groups in B ring is necessary for strong inhibition of α -glucosidase (Fig 1). Tadera *et al.*, (2006) have studied the inhibition of α -glucosidase and α -amylase by natural flavonoids and has shown that the presence of hydroxyl group at C-3 position of flavone enhanced the inhibitory activity [quercetin>luteolin; kaempferol>apigenin] and they have also reported that the presence of more than two hydroxyl groups in B ring inhibits α -glucosidase more effectively.

Figure 1: Structures of the isolated compounds from *Psidium guajava* leaves and *syzygium cumini* Linn seed and related flavonoids with substituent groups at various positions



3,5,3',4' – OH ; 7- Glu: Dihydro-3,3',4',5,7 pentahydroxy flavone glycoside
 4',5,7- OH; 7- Glu: Apigenin-7-O-glucoside
 3',4',5,7 – OH: Luteolin
 5,7,4' – OH: Kaempferol
 3,5,7,3',4' – OH : Catechin
 5,6,7 – OH: Baicalein

Effect of hydroxyl group at various positions at A ring has been studied by Gao *et al.*, (2004). They showed that the presence of hydroxyl group at C-5, 6 & 7 positions alone has significant α -glucosidase inhibitory activity whereas others have little or no inhibition. It has been known that the substituent groups of the flavones have a great impact on the enzyme inhibition activity. C-3'OH of B ring of flavones in particular increase the α -glucosidase inhibitory activity whereas C-glycosylation at C-6 or C-8 of A-ring weakened the inhibition activity (Li *et al.*, 2009).

In the present study, inhibition of α -glucosidase by dihydro-3,3',4',5,7-pentahydroxy flavone may be due to the presence of two OH groups in the B ring and the glucoside substitution might have also an positive effect on the α -glucosidase inhibition. Other compound, apigenin-7-O-glucoside identified in our study lack C-3'OH group however, it shows significant α -glucosidase inhibition indicating that the presence of glucoside substitution at C-7 position, have favoured the inhibition and might have played an significant role in inhibition of the α -glucosidase.

4. CONCLUSION

We screened for low molecular weight α -glucosidase inhibitors from various traditional plant parts used for the treatment of diabetes, of which maximum inhibition was exhibited by *S.cumini* seed extracts and *P. guajava* leaves and active component inhibiting α -glucosidase were found to be apigenin 7-O-glucoside and dihydro-3,3',4',5,7 – pentahydroxyflavone glycoside respectively. Since, the present investigation does not involve any *in-vivo* studies, the observed inhibitory activity towards α -glucosidase by these plant extracts (Table 1) might be one the possible reason for their hypoglycemic activity.

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