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# Identification of α amylase inhibitors from *Syzygium cumini* Linn seeds

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The aqueous extract of *S. cumini* or *Eugenia jambolana* seeds and *Psidium guajava* leaves showed higher inhibition against the porcine pancreatic α-amylase among the medicinal plants studied. The α-amylase inhibitors from *S.cumini* seeds were separated from the extract by preparative thin layer chromatography into fractions with different Rf values. The fraction with $R_f$ value between 0.285 and 0.43, which showed maximum inhibitory activity, was eluted and analyzed through LC-MS. The compounds identified from the seed extract of *S. cumini* were betulinic acid and 3,5,7,4`-tetrahydroxy flavanone, which were reported earlier from *S. formosanum* and other plants. Dixon plot showed that the inhibition was non-competitive in nature.

**Keywords**: Amylase inhibitor, Diabetes mellitus, Non-competitive inhibitor, Porcine pancreatic α-amylase, *Syzygium cumini*

It is estimated that more than 200 species of plants exhibit hypoglycemic properties[1-5]. One of the extensively studied plants is *Syzygium cumini*. The mice treated with the fruit pulp and seed extract showed a significant decrease in blood glucose level[6-8]. However, no study was carried out on the amylase inhibitory activity of *Syzygium cumini* seeds. Pancreatic α- amylase hydrolyses starch to maltose and oligosaccharides in the small intestine, whereas, membrane bound intestinal α-glucosidase hydrolyses di-and oligosaccharides to glucose. Inhibition of these enzymes reduces the rate of digestion of starch and result in a decrease in the post-prandial blood glucose levels in diabetic patients. The present investigation was initiated to screen *S.cumini* and other antidiabetic plants for α- amylase inhibitory activity and to isolate, purify and to identify the compounds showing α- amylase inhibitor activity.

## Materials and Methods

Plant materials—Medicinal plants *Zizyphus mauritiana*, *Aegle marmelos*, *Murraya koenigii*, *Gymnema sylvestre*, *Syzygium cumini*, *Limonia acidissimia*, *Tinospora cordifolia*, *Acalypha indica*, *Trigonella foenum graecum*, *Moringa oleifera*, and *Psidium guajava* were chosen for the experiment because these plants have been recommended as herbal medicine for the treatment of diabetes in Ayurvedic system of medicine and used in India and other countries. Compounds with antidiabetic activity have been purified and even patented from some of the above plants[2-5]. The medicinal plants used in the present study were collected from Tamil Nadu Agricultural University (TNAU) orchard, Coimbatore, India and authenticated by the Professor, Medicinal Plants,TNAU,Coimbatore.

*Preparation of extracts of plants*—The medicinal plant parts (shown in Table 1) were sun dried and ground to a fine powder and stored at room temperature. The dried powder (5 g) was extracted with a mixture of 22 ml of acetone and 3 ml of petroleum ether in a clean dry mortar and pestle as described by Nassar *et al* [9] and Venkat Reddy *et al*[10]. The filtrate was poured into a separating funnel containing 20 ml of petroleum ether and 20 ml of 10% NaCl. The petroleum ether layer was separated and discarded. The aqueous layer was again extracted with ethyl acetate and the organic phase was discarded. After evaporation of organic solvents in the aqueous phase it was made up to 25 ml with water. The aqueous phase was used as source of α-amylase inhibitor assay.

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α-Amylase inhibitory activity of each extract was analyzed by the method of Bernfeld[11] with a little modification as described below. In brief, 100 µl of the test extract was allowed to react with 200 µl of the porcine pancreatic α-amylase enzyme (Sigma Aldrich-3176) and 100 µl of 2 mM of phosphate buffer (*p*H 6.9). After 20 min incubation, 100 µl of 1% starch solution was added. The same was performed for the control where 200 µl of the enzyme was replaced by buffer. Enzyme working standard was prepared by dissolving 1 mg of porcine pancreatic amylase in 10 ml of phosphate buffer (*p*H 6.9). After incubation for 5 min, 500 µl of dinitrosalicylic acid reagent was added to both control and tests. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm using a spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula:

Inhibition (%) = 100 (control-test/control)

Suitable reagent blank and inhibitor controls were simultaneously carried out and subtracted.

Dose dependent variation in the α-amylase inhibitory activity was measured using 25 to 125 µl of aqueous extract.

*Separation and purification of compounds*—The aqueous extract was subjected to thin layer chromatography using silica gel-G. Different solvent systems were employed for the separation of compounds in the extract. Identification of phenolics, terpenoids and alkaloids was done by spraying with Folin-Ciocalteau reagent, vanillin-sulphuric acid and Dragendorffs reagent, respectively. The preparative TLC plate was allowed to run with the solvent system hexane, ethyl acetate and acetic acid in the ratio of 20:20:10, respectively. The developed plate was divided into different fractions based on $R_f$ values and each fraction was tested for α-amylase inhibitory activity. The fraction, which showed the maximum inhibition for α-amylase, was analyzed through LC-MS available at the Regional Sophisticated Instrumentation Centre, Central Drug Research Institute, Lucknow, India. The ESI-LC-MS was recorded on a Micromass Quattro triple quadrupole mass spectrometer having a Jasco Pu-980 HPLC Pump connected to it. The column was Waters Sphersorb ODS-2 (250 × 4.6mm × 5µ). The solvent system was acetonitrile:water, 70:30 containing 0.1% formic acid. The photodiode array detector was monitored at 200-650 nm.

## Results

Among the 11 medicinal plants tested against porcine pancreatic α-amylase, *Syzygium cumini* seeds and *Psidium guajava* leaves showed strong inhibition (98%). The inhibition obtained in other medicinal plants ranged from 0 to 20% (Table 1). Since *S. cumini* seeds are widely used for antidiabetic activity in Ayurvedic and Homeopathic medicines, it was selected for further study.

The inhibitory secondary metabolites present in the aqueous phase of the acetone plus petroleum ether extracts of the tested medicinal plants belong to hydrophilic group. Percentage inhibition was tested with different concentrations (25-125 µl) of the aqueous phase, with fixed substrate concentration. Though percentage inhibition increased slightly from 79% to 91% with increased in volume of the extract from 25 to 75 µl, further increase in volume to 125 µl resulted in negligible increase. The type of inhibition of these inhibitors on porcine pancreatic α-amylase was found to be non-competitive in nature as characterized using Dixon plot.

Preparative thin layer chromatography was performed after concentrating the aqueous phase of the petroleum ether plus acetone extract of *S. cumini* seed (2 ml). Spraying the TLC plates with Folin-Ciocalteau reagent, vanillin-sulphuric acid and

Table 1— Inhibition of porcine pancreatic α-amylase by the extracts from medicinal plants

| S.No | Medicinal plants | Plant parts used | Porcine pancreatic α-amylase inhibition (%) |
|---|---|---|---|
| 1 | *Zizyphus mauritiana* | Seeds | 12 |
| 2 | *Limonia acidissimia* | Seeds | 20 |
| 3 | *Tinospora cordifolia* | Leaves | 13 |
| 4 | *Acalypha indica* | Leaves | 15 |
| 5 | *Syzygium cumini* | Seeds | 98 |
| 6 | *Psidium guajava Var. Pomiferum* | Leaves | 98 |
| 7 | *Aegle marmelos* | Leaves | 6 |
| 8 | *Murraya koenigii* | Leaves | No inhibition |
| 9 | *Gymnema sylvestre* | Leaves | 3 |
| 10 | *Trigonella foenum graecum* | Seeds | 10 |
| 11 | *Moringa oleifera* | Leaves | 16 |

Table 2 — Porcine pancreatic α-amylase (PPA) inhibition per cent of TLC fractions from *Syzigium cumini* seed extract.

| Fraction number | $R_f$ Value | PPA inhibition (%) |
|---|---|---|
| 1 | 0 – 0.140 | 92 |
| 2 | 0.141 – 0.284 | 91 |
| 3 | 0.285 – 0.430 | 98 |
| 4 | 0.431 – 0.570 | 92 |
| 5 | 0.571 – 0.700 | 91 |
| 6 | 0.710 – 0.860 | 92 |
| 7 | 0.861 – 1.00 | 65 |

Dragendorffs reagent, identified the presence of phenolics, terpenoids and alkaloids, respectively in the extract.

The fraction with $R_f$ value 0.285 to 0.43 in the preparative TLC, which inhibited 98% of the α-amylase activity (Table 2), was separated using RP-HPLC. The mass spectral analysis of the HPLC purified sample revealed the presence of betulinic acid and 3,5,7,4`-tetrahydroxy flavanone. The molecular ion peak of betulinic acid was found to be at m/z 454 and other peaks at m/z 453 $(M-H)^+$ and m/z 437 $(M-OH)^+$ were noticed. The molecular ion peak of 3,5,7,4' – tetrahydroxy flavanone was found at m/z 288.

## Discussion

In the present study, among the medicinal plants tested, *S. cumini* seeds and *P. guajava* leaves showed strong inhibition (98%) of α-amylase activity. Studies with the *S.cumini* leaf extract on glycaemia of diabetic and non-diabetic mice by Sharma *et. al*[7,8] and Oleiveira *et. al*[12] showed that crude ethanolic and aqueous extracts reduced glycaemia of diabetic mice and some compounds prepared from the extracts have been patented by them[7,8]. The oral glucose tolerance test showed anti-hyperglycemic activity of the bark of *S. cumini* when fed simultaneously with glucose[6]. The present study was planned to identify α-amylase inhibitor having the potential of antidiabetic activity in *S. cumini* seeds.

Yoon and Robyt[13] examined the inhibition kinetics of acarbose and its two analogues, 4IV–maltohexaosyl acarbose and 4IV–maltododecaosyl acarbose on α-amylases from four different sources: *Aspergillus oryzae, Bacillus amyloliquefaciens*, human saliva and porcine pancreas. The three inhibitors showed mixed, noncompetitive inhibition, for all four α-amylases. A similar type of inhibition for *S. cumini* seed extract as evidenced by the Dixon plot was also obtained.

The LC-MS data was used to identify the compounds in the TLC separated fraction. Based on the molecular ion peak, fragmentation pattern and the data available in literature, the compounds were identified as betulinic acid and 3,5,7,4`-tetrahydroxy flavanone. Betulinic acid was already reported from *Syzygium formosanum*[14]. The presence of flavonoids in *S. cumini* seeds was reported by Teixeira *et. al*[15]. Flavonoids such as apigenin-7-O-glucoside inhibited α-amylase activity[16]. Luteolin, myricetin and quercetin flavonoids were reported as potent inhibitors of porcine pancreatic α-amylase with the $IC_{50}$ values less than 500 μ*M*[17]. McDougall *et. al*[18] tested the polyphenol rich extracts from soft fruits for their ability to inhibit α-amylase and α-glucosidase. Kandra *et. al*[19] studied the inhibition of tannic acid on human salivary amylase.

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* study using Gato- Kakazaki (GK) rats[20]. The authors predicted that the inhibition of α-glucosidase by *S.cumini* seed extract as a possible mechanism for the antidiabetic action. Since the extract as well as the TLC purified fraction obtained in the present study showed strong α-amylase inhibitory activity, further experiments are necessary to find out whether the extract possesses antidiabetic activity under *in vivo* conditions. Though the TLC separated compounds showed high α - amylase inhibitor activity, the inhibitory activity of the individual compounds in *S. cumini* seeds has to be test verified.

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