Effect of Sprouting Time on the alpha Amylase Inhibitory Activity of Fenugreek *(Trigonella foenum graecum L.)* Seeds

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ABSTRACT

Alpha amylase, an enzyme which helps in digestion of starch and glycogen. Inhibition of this enzyme holds off carbohydrate metabolism and extends the total carbohydrate digestion time, thereby reducing the postprandial plasma glucose rise. Fenugreek (Trigonella foenum graecum L.) has been commonly used as a traditional medicine and spice. It is known to have hypoglycemic effects. The objective of this study was to evaluate the effect of sprouting time on alpha amylase inhibitory activity of fenugreek seeds germinated over a 10 days period. Germinated seeds were dried, powdered, and stored at 4° C. Acarbose, an anti-diabetic drug was used for comparing our results. In vitro inhibitory alpha amylase assay was carried out for the aqueous seed extracts and for Acarbose. IC_{50} values were also determined.

The germination process influenced the amylase inhibitory activity of fenugreek seeds with the maximum percentage of inhibition obtained on 3rd day of sprouting (38.2%) for aqueous extract. Acarbose showed 31.7% amylase inhibition. An IC_{50} assay of 3rd day fenugreek extract gave a value of 19.87 mg/ml, while for Acarbose an IC_{50} value of 4.33 mg/ml was obtained. These studies indicate that germinated seed extracts have higher potential for usage as a pharmaceutical agent for treating hyperglycemia.

Keywords: *Alpha amylase, Fenugreek, Sprouting time, Alpha amylase inhibitory activity, Acarbose.*

1. Introduction

Fenugreek (Trigonella foenum graecum L.) is a legume which is used as a spice in many parts of the world to enhance the overall sensory qualities of foods. The plant is widely grown in South Asia, North Africa and parts of the Mediterranean region. In our ancient Ayurveda system, the seeds of this plant have been popular for its various medicinal properties such as – antidiabetic, anticarcinogenic, hypocholesterolemic, antioxidant and immunological activities. Besides its medicinal value, fenugreek is also utilized for food product development as food stabilizer, adhesive, and emulsifying agent (Wani & Kumar, 2018). The leaves of this plant also possess multiple essential vitamins and minerals. The seeds of fenugreek have been used orally as an insulin substitute for reduction in blood glucose, and the extracts from seed have been reported to lower blood glucose levels (Madar & Stark, 2002).

Diabetes mellitus has been one of the most notorious metabolic disorders for years. The characteristic feature of this disorder is inappropriately high levels of blood glucose or hyperglycaemia. Mainly, there are two subtypes, Type I Diabetes mellitus (T1DM) and Type II Diabetes mellitus (T2DM) and gestational diabetes. The two subtypes classically result from defective insulin secretion (T1DM) and/or action (T2DM). Both of these types have very different pathogenesis, etiologies, clinical presentations and are dealt with different treatment approaches too. In both type 1 and type 2 diabetes, various genetic and environmental factors can result in the progressive loss of β-cell mass and/or function that manifests clinically as hyperglycaemia. Once hyperglycaemia occurs, patients with all forms of diabetes are at risk for developing the same chronic complications, although rates of progression may differ (American Diabetes Association, 2020). Consequently, the chronic metabolic derangement can affect all the vital organs of the body, contributing to the risk of cardiovascular diseases and other complications like- retinopathy, nephropathy or neuropathy. The treatment of DM is achieved through parenteral insulin and oral antidiabetic drugs. Oral hypoglycaemic agents include sulphonylureas, biguanides and other drugs like acarbose. These drugs have serious side effects and deleterious contraindications (Wadkar et al., 2007). Long term usage of insulin and hypoglycaemic agents leads to increase blood sugar, drug resistance, adverse effects and complications which will further affect the immune system of the body. To avoid such problems, it seems beneficial to

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use ayurvedic formulations for better management of diabetes mellitus (Mishra, 2003).

Herbal medicines with anti-diabetic potential have different modes of action- mimic insulin, act on insulin secreting beta cells, or modify glucose utilization. Herbs which modify glucose utilization act by altering the viscosity of gastrointestinal contents, delaying gastric emptying, or delaying glucose absorption (Wadkar et al., 2007).

The α-amylase (α-1, 4-glucan-4-glucanohydrolase) is one of the major secretory products of pancreas and salivary glands, which helps in digestion of starch and glycogen. Rapid degradation of dietary starch by α-amylase leads to elevated postprandial hyperglycemia (PPHG) (Kamtekar et al., n.d.). Inhibition of this enzyme holds off carbohydrate metabolism and extends the total carbohydrate digestion time, thereby reducing the postprandial plasma glucose rise.

Therefore, plant based α-amylase and α-glucosidase inhibitors are likely to be useful in regulating blood-glucose level. T. foenum-graecum has demonstrated α-amylase and α-glucosidase inhibitory potential which may serve as a lead for isolation and identification of compounds responsible for it (Ganeshpurkar et al., 2013). Fenugreek has been shown to have a high efficacy at relatively low doses and during both acute and longer-term interventions (Madar & Stark, 2002).

Germination or sprouting is a processing intervention by which nutritional content of the crop can be enhanced significantly (Pandey & Awasthi, 2015). Also, germinated fenugreek seeds have higher antioxidant content and enhanced antidiabetic effect than its boiled counterpart (Madhava Naidu et al., 2011). So, the present study is aimed at investigating the effect of germination on alpha-amylase inhibitory activity of fenugreek seeds for its development as a nutraceutical for treating diabetes effectively without side-effects. The results would then be compared with a commonly used anti-diabetic drug-Acarbose (trade names – Glucobay, Precose, or Prandase).

2. Materials and Equipments

(a) Fenugreek seeds obtained from the local market of Delhi/NCR region of India.

(b) Chemicals used included 3,5-dinitro salicylic acid (DNSA) reagent, sodium potassium tartrate tetrahydrate, sodium hydroxide, 1% starch solution, sodium phosphate buffer (pH 6.9), sodium chloride, α-amylase enzyme (0.5 mg/ml).

(c) An anti-diabetic drug was also used - Acarbose.

(d) Equipments used were oven, vortex, spectrophotometer and water bath.

3. Methodology

3.1 *Preparation of extract, buffer, reagents and standard solutions*

Fenugreek seed soaking and germination: Fenugreek seeds in ten portions of 50 g each were soaked in 70% ethanol solution for 15 minutes at room temperature for disinfection. Soaked seeds were then washed with tap water and distilled water. Washed seeds were then soaked in distilled water (1:10 w/v) for 12 h at room temperature. The pre-soaked seeds were again washed in distilled water and kept for germination on flat trays lined with moist paper towel. The trays were covered with aluminium foil for dark germination. The germinating seeds were kept moist with distilled water and germinated for 10 days. Sprouted seeds after each day starting from first day were frozen to stop further germination. After thawing at room temperature, seeds were dried in an electric air draught oven at 60°C for 48 hrs. Dried and sprouted seeds were ground in an electric grinder, sieved and stored in plastic bottle container at 4°C for further analysis (Jain et al., 2017).

Stock solutions of extracts: Extracts of germinated seeds of each day were prepared by dissolving 10g powder in 50ml distilled water (20% w/v). This was lyophilized and then assays were carried out with freshly prepared solutions.

0.02 M Sodium phosphate buffer with 6.7mM sodium chloride: This buffer was prepared by mixing 0.02 M disodium hydrogen phosphate, 0.02 M sodium dihydrogen phosphate and 6.7 mM NaCl. The pH was adjusted till 6.9.

DNSA Reagent preparation: 1 g of 3, 5-dintro salicylic acid (DNSA) was dissolved in 50 ml of distilled water and then 30 g of sodium potassium tartrate was added in small lots. To the above, 20 ml of 2 N sodium hydroxide was added; it turned into transparent orange yellow colour. Volume was made up with 100 ml of distilled water. The reagent bottle was wrapped in brown paper and stored in dark and cool place.

1% starch solution: 0.2g of starch was added to 20 ml of 0.02 M Sodium phosphate buffer and then this was boiled to dissolve the starch. The solution was cooled and stored at 4°C. Prior to the assay this was incubated at 25°C for 5 minutes.

α-amylase enzyme solution: 50 mg of α-amylase powder was dissolved in pre-chilled 0.02 M sodium phosphate buffer to yield a concentration of 0.5mg/ml solution. This was then incubated at 25°C for 10 minutes prior to the assay.

3.2 In-vitro inhibitory anti-amylase assay

The 500 µL of fenugreek seed extract was incubated with 500 µL of α-amylase solution (0.5 mg/ml) at room temperature (32°C) for about 10 minutes. After incubation,

500 µL of 1 % starch solution was added and was incubated at room temperature (32°C) for about 10 minutes. To the above, 1 ml of DNSA reagent was added to stop the reaction and was incubated in hot water bath (85°C) for 5 minutes. After 5 minutes, the reaction mixture colour changed to orange-red and was removed from water bath and cooled to room temperature (Kamtekar et al., n.d.) [5]. It was diluted to 10 ml with distilled water. Extracts for each germination day were performed in triplicates. Individual blank was performed by replacing enzyme with buffer. Control was performed by using the enzyme without the extracts. Absorbance was measured at 540 nm using a spectrophotometer. (Saxena & Jain, 2018).

The inhibition percentage of α-amylase was assessed by the following formula:

The α-amylase inhibitory activity =

$$\frac{(Ac^{+}) - (Ac^{-}) - (A_s - A_b) \times 100}{(Ac^{+}) - (Ac^{-})}$$

Where,

Ac^{+} is the absorbance of 100% enzyme activity (only solvent with enzyme)

Ac^{-} is the absorbance of 0% enzyme activity (only solvent without enzyme)

A_s is the absorbance of test sample with enzyme

A_b is the absorbance of test sample without enzyme.

The α-amylase inhibition percentages for all germination days were determined using this assay. Acarbose was analysed with this method too.

3.3 *IC_{50} analysis for in-vitro inhibitory anti-amylase assay*

After determining the germination day at which the alpha-amylase inhibition percentage was maximum, IC_{50} analysis was done for that particular extract. Extracts at different concentrations (2, 5, 10, 15, 20 mg/ml) were performed in triplicates. Individual blank was performed by replacing enzyme with buffer. Control was performed by replacing extract with solvent. Absorbance was measured at 540 nm in a spectrophotometer. A graph was established by plotting percentage of alpha amylase inhibition against sample concentration in order to calculate IC_{50} (inhibitory concentration) value. The obtained value represents sample concentration (mg/ml) required to decrease the activity by 50 % of α-amylase.

3.4 *IC_{50} analysis for Acarbose*

The anti-diabetic drug acarbose was crushed into a fine powder. Acarbose powder at different concentrations (4, 10, 20, 30, 40 mg/ml) were performed in triplicates. Individual blank was performed by replacing enzyme with buffer. Control was performed by replacing the drug with solvent. Absorbance was measured at 540 nm in a spectrophotometer. IC_{50} graph was plotted for this drug in the same way as for the fenugreek extract.

4. Results and Discussion

4.1 *Determination of alpha amylase inhibition activity*

The fenugreek aqueous seed extract for 3rd day of germination gave the highest percentage of alpha amylase inhibition activity. It was found to be 38.5%. Further, it was observed that the alpha amylase inhibitory activity of seed extracts gradually increased from day 0 to day 2 then finally peaking at day 3, but after day 3 the inhibition percentages start to decrease and is reduced to a minimum at day 10. So, an IC_{50} assay was carried out for Day 3 extract.

4.2 *Determination of IC_{50} value for Day 3 Fenugreek aqueous extract*

The IC_{50} value for Day 3 Fenugreek aqueous extract was found out to be 19.87 mg/ml. This value indicates that for achieving 50% alpha amylase inhibition 19.87 mg/ml of the seed extract is required. Alpha amylase inhibition results in reduction of dietary starch hydrolysis, thereby contributing to decreased postprandial glucose rise. So, it indicates that sprouted fenugreek seeds are more effective for treating hyperglycaemia in diabetic patients.

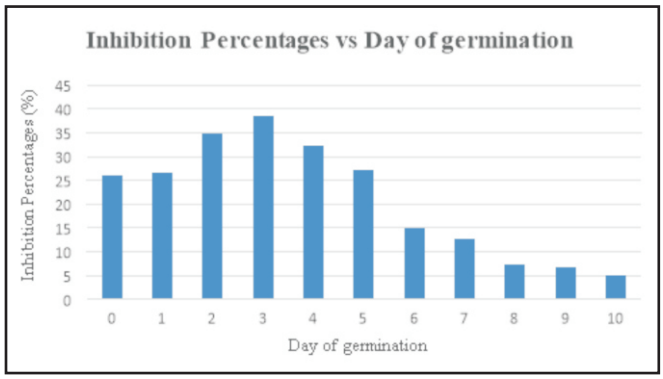

Fig. 1: *Bar graph showing alpha amylase inhibition activity of fenugreek aqueous seed extract from Day 0 to Day 10 of germination.*

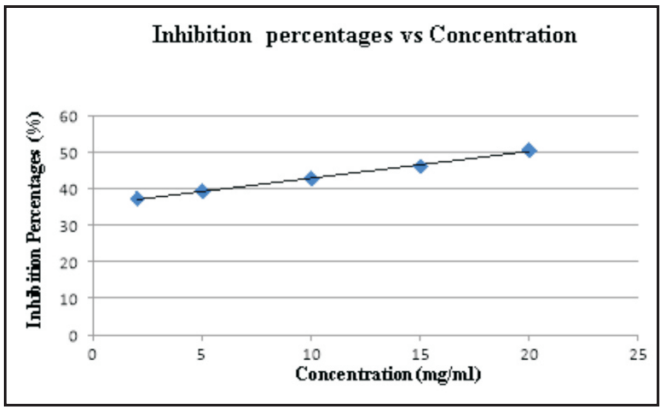

Fig. 2: *Graph for IC50 assay of Day 3 fenugreek aqueous extract*

4.3 *Determination of IC_{50} value for anti-diabetic drug acarbose*

The IC_{50} value for Acarbose was found to be 4.33 mg/ml. This value represents the concentration of the drug required to achieve 50% alpha amylase inhibition. Acarbose is actually a starch blocker, it inhibits intestinal alpha-glucosidase and pancreatic alpha-amylase needed for hydrolysing carbohydrates that releases glucose. The IC_{50} value is lower than the sprouted fenugreek seed extract but, it also has many side effects which is disadvantageous for the diabetic patients. Side effects include gastrointestinal problems, hypoglycaemia, and hepatotoxicity which might lead to acute hepatitis in patients treated with acarbose (Diaz-Gutierrez et al., 1998).

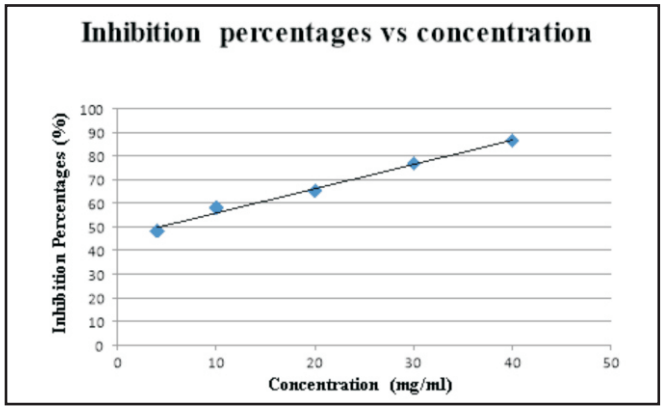

Fig. 3: *Graph for IC50 assay of Acarbose*

5. Conclusion

The results obtained from the above study highlights the nutraceutical power of sprouted fenugreek seeds. The anti-amylase inhibitory activity increased from day 0 to day 3 of germination, giving the maximum activity on day 3 i.e., 38.5%. Thereafter, this activity of fenugreek seeds reduced. An IC_{50} assay of 3rd day fenugreek extract gave a value of 19.87 mg/ml, while for Acarbose an IC_{50} value of 4.33 mg/ml was obtained. Comparing both these values, it is obvious that the dosage of acarbose require for achieving 50% amylase inhibition is less than the fenugreek seed extract. This might appear advantageous, but consumption of acarbose and other anti-diabetic drugs poses many adverse side effects and health risks. This can be avoided by adopting natural alternatives like sprouted fenugreek seed extract, which is much more cost-effective and has no such side effects. Thus, sprouted fenugreek seeds can be used as a nutraceutical and further analysis can give rise to the bioactive molecules responsible for this amylase inhibitory activity. This can then be used to isolate lead compounds for designing a new array of anti-diabetic drugs.

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