In-vitro Inhibitory Effects of Polyphenolic Extract of *Ichnocarpus Frutescens* on Carbohydrate Digestive Enzymes

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Abstract Background: *Ichnocarpus frutescens* has been used as Ayurvedic herbal medicine by Gond tribes in India to treat several diseases such as diabetes mellitus and Jaundice. The growing incidence of diabetes mellitus and its complications have headed to the search of novel therapeutic approaches engrossed on preventing postprandial hyperglycemia. The practice of carbohydrate breakdown enzyme inhibitors from plant resources might be a potential approach to prevent glucose absorption with the least adverse effects. Aims: The objective of the present investigation was to provide in-vitro evidence for the potential inhibitory activity of polyphenolic extract (PPE) of *Ichnocarpus frutescens* on α -amylase and α -glucosidase enzymes. Methods: the in the vitro inhibitory effect of polyphenolic extract (PPE) of *Ichnocarpus frutescens* focused on the evaluation of pancreatic α - amylase inhibition, rat serum α - amylase inhibition and rat intestinal α -glucosidase inhibition of polyphenolic extract (PPE) of *Ichnocarpus frutescens* by *in vitro*. The different concentrations of polyphenolic extract were subjected to α -amylase and α -glucosidase inhibitory assay and the percentage of α - amylase and α - glucosidase inhibitory activity and IC₅₀ values were calculated. Results: Polyphenolic extract shows appreciable pancreatic α -amylase inhibitory activity *in vitro*. The extract also showed an appreciable α -glucosidase inhibitory effect in a concentration-dependent manner with a moderate α -amylase inhibitory activity. The *in vitro* examination of the inhibitory effect of PPE on maltase and sucrase activities revealed that PPE inhibited rat small intestine disaccharidase (α -glucosidase) activity. Conclusion: Taken together, these results suggest that inhibitory effect of PPE on α - amylase and α - glucosidase activities may delay

carbohydrate digestion and absorption with subsequent lowering of blood glucose level leading to prevention of postprandial hyperglycemia in diabetes and its complication.

Keywords *Ichnocarpus Frutescens* Extract, Carbohydrate Metabolism, α -amylase, α -glucosidase, Diabetes Mellitus, Postprandial Hyperglycemia

1. Introduction

Hyperglycemia is a condition characterized by an abnormal excess of sugar in the blood. Chronic postprandial hyperglycemia is widely recognized as one of the earliest disease biomarkers in the prediction of subsequent microvascular and macrovascular complications that can progress to full symptomatic Type2 diabetes mellitus [1-2]. The frequency of diabetes in the population worldwide is high and still increasing. In the process of this metabolic disorder, concentrations of blood glucose and serum insulin differ from normal concentration, especially after food intake. While the majority of available synthetic antidiabetic drugs target the dual metabolic effects that characterize type II diabetes, impaired insulin secretion, insulin resistance, some of these drugs can have negative side effects at high doses [3-4]. An effective way to control these carbohydrate dependent diseases would be to limit the intestinal carbohydrate digestion.

Starch and sucrose account for 80 % to 90 % of our daily intake of carbohydrates. Small intestinal α -glucosidase

(EC.3.2.1.20) and pancreatic α -amylase (EC.3.2.1.1) are key enzymes of dietary carbohydrate digestion in humans. Inhibitors of these enzymes may be effective in retarding carbohydrate digestion and reduce glucose absorption not to support postprandial hyperglycemia. Plants have been used to treat diabetes mellitus since ancient times. Studies to reveal the mode of action of potential anti-diabetic plants will definitely confer scientific and systematic approach to the use of these plants as hypoglycemic agents. Findings from recent clinical and preclinical studies suggest that plant derived-polyphenolic foods could become valuable treatment options for optimizing hyperglycemic control in diabetic patients. The relative efficacy of α -amylase inhibitors in affecting human digestion or starch has been highlighted by recent publications [5].

α -amylase and α -glucosidase inhibitors are drug design targets for the developments of diabetes, obesity, and hyperglycemia [6]. Inhibitory activity against α -amylase, and glucosidase by polyphenols, flavonoids, and anthocyanins has been reported [7-8]. In addition, numerous α -glucosidase and α -amylase inhibitors have been screened from plants, some of which are of clinical importance [9-10]. α -glucosidase inhibitors such as acarbose, miglitol, and voglibose are known to reduce postprandial hyperglycemia primarily by interfering with the carbohydrate digestive enzymes and delaying glucose absorption. Therefore, much effort has been focused on plants for potentially useful products as commercial α -amylase and α -glucosidase inhibitors.

Plants belonging to Apocynaceae family have been used for the common ailments as traditional medicine. Recent research has now indicated that these herbs are a rich source of phenolic phytochemicals having high antioxidant activity. Phenolic phytochemicals are now implicated to have the potential for the management of many chronic diseases such as diabetes and cardiovascular diseases [11]. Consequently, *Ichnocarpus frutescens* has gained prominence as a functional food for the treatment of diabetes among tribals of several Indian regions [12]. The health-promoting effects of *I. frutescens* are thought to be related to the high polyphenolic content. Distribution of various flavonoids and phenolic acids in the leaves of *Ichnocarpus frutescens* have been systematically studied [13]. In the present study, the polyphenolic extract (PPE) of the *I. frutescens* was examined for its *in vitro* inhibitory effect on rat intestinal α -glucosidase, α -amylase (Porcine Pancreatic amylase) and rat serum α -amylase activities.

2. Materials and Methods

2.1. Chemicals

α -amylase, folin-ciocalteu reagent, precoated with silica gel 60F₂₅₄, *p*-anisaldehyde reagent, fast blue B salt, pyrocatechol, starch, dinitrosalicylic acid colour reagent, 2-chloro-*p*-nitrophenyl- α -D-maltotrioxide (CNPG3), 2, 4 –

dinitrophenylhydrazine, maltose, sucrose, naringenin, quercetin, pentobarbitone were purchased from Sigma Aldrich, Bangalore, India. All other chemicals and solvents of analytical grade were purchased from local.

2.2. Plant Materials

The fresh leaves of *Ichnocarpus frutescens* (L.) R.Br. recently were collected from Delta region of Cauvery River, Thiruchirappalli, India and was authenticated at Botanical Survey of India, Central National Herbarium, Howrah, India (REF NO: CNH/I-I/87-TECH/1326). An authentic voucher specimen was deposited in the Herbarium Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

2.3. Extraction of Polyphenols (PPE)

Dried leaves of *Ichnocarpus frutescens* (500 g) were finely powdered, mixed with 70% methanol and kept at room temperature for 5 days. After 5 days it was filtered and the solvent was evaporated. The residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 mL/L). Extraction of polyphenols was carried out for 36 h at room temperature and the combined ethyl acetate layer was concentrated. The residue was lyophilized and stored at -70°C. The total polyphenolic content and flavonoid of the extract were assayed using the standard methods.

2.4. Animals and Ethical Approval

Swiss albino mice (20 - 25g body weight) and Wistar albino rats (180 - 200g body weight) were used in the present study. Animals were collected from the breeding colony and acclimatized to the laboratory condition for 2 weeks. They were housed in macrolon cages under standard laboratory conditions (light period from 7.00 a.m. to 7.00 p.m., temperature 21 $\pm$ 2°C, and relative humidity 55-70 %). The animals were fed with commercial diet from Hindustan Lever Ltd (Bangalore, India) and free access to water (*ad libitum*) during the experiments.

2.5. α -amylase Inhibitory Activities

2.5.1. α -amylase Inhibitory Assay by DNS Method (*in vitro*)

Porcine pancreatic α -amylase (EC 3.2.1.1) was purchased from Sigma Aldrich Chemical Co. A total of 500 μ L of PPE and 500 μ L of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α -amylase solution (0.5 mg/mL) were incubated at 25°C for 10 min. After pre-incubation, 500 μ L of a 1% starch solution in

0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 mL of dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted by adding 10 mL distilled water and absorbance was measured at 540 nm. The bioassay method was adopted and modified from Sigma Aldrich (www.sigmaaldrich.com).

Inhibition =

$$\frac{\text{Abs}_{\text{control}}(540) - \text{Abs}_{\text{extract}}(540)}{\text{Abs}_{\text{control}}(540)} \times 100\%$$

2.5.2. α -amylase Inhibitory Assay by CNP-G₃ Method (*in vitro*)

The ability of porcine pancreas α -amylase to release 2-chloro-4-nitrophenol (CNP) from CNP-G₃ was measured with a slight modification of the methods described previously [14]. A reaction mixture (450 μ L) consisting of 0.15 mM CNP-G₃ and 0.2 M potassium thiocyanate (KSCN) solution in 0.05 M phosphate buffer pH 7.0, and 30 μ L of different concentrations of PPE were pre-incubated for 5 min at 25 °C followed by the addition of 20 μ L of freshly prepared α -amylase solution (1 mg/mL) in phosphate buffer, pH 7.0. Liberation of CNP from CNP-G₃ was determined by measuring the increase in absorbance at 405 nm during the reaction. The inhibitory activity (%) was calculated as $[(A-B)/A] \times 100$, where A is the increase absorbance during the reaction mixture in the absence of PPE and B is that in its presence.

2.5.3. α -amylase Inhibitory Assay by Starch Iodine Method (*in vitro*)

The activity α -amylase was carried out according to the previously described method, based on starch-iodine colour changes with minor modification [15]. 0.1 mL of α -amylase (15 μ g/mL in 0.1 M acetate buffer at pH 7.2 containing 0.0032 M sodium chloride) was added to the mixture of 3 mL of 1% starch solution. The solution was kept in the refrigerator and was used within 3 days and 2 mL of acetate buffer (0.1 M, pH 7.2) pre-equilibrated at 30°C in a water bath. The substrate and α -amylase blank determinations were undertaken under the same conditions. At zero time (t=0 min) and at the end of the incubation period (t = 60 min), 0.1 mL of the reaction mixture was withdrawn from each tube after mixing and transferred into 10 mL of an iodine solution (0.254 g iodine and 4.0 g potassium iodide in 1 L). After mixing, the absorbance of

the starch-iodine mixture was measured immediately at room temperature at 565 nm.

2.5.4. Inhibition of Rat Plasma α -amylase Activity (*invivo*): Preparation of Rat plasma

Male Swiss albino rats (4-6weeks) were maintained under standard environmental conditions with 12 h light and 12 h dark exposure. The animals were allowed free access to food and water. Investigations using experimental animals were conducted in accordance with internationally accepted principles of laboratory animal use and care. After overnight fasting, the rats were sacrificed and cardiac blood was collected from the rat with a heparin-treated cylinder and centrifuged at 5000 $\times$ g for 20 min. The plasma was collected and stored at 4° C until further use.

2.5.6. α -amylase Assays in Rat Plasma

α -amylase activity was carried out according to the good-starch method [16]. Briefly, 0.5 mL of substrate buffer solution (0.25 M phosphate buffer at pH 7.0 containing 40 mg/dL soluble starch) was mixed well with 0.10 mL of distilled water and kept at 37°C for 5 min. this was done for both sample and blank. After the incubation period, 0.01 mL of rat plasma was added to the sample solution only and well mixed before being re-incubated at 37° C for 7.5 min. after the second incubation time, 0.5 mL of colouring agent (0.254 g iodine and 4.0 g potassium iodide in 1 L) and 2.5 mL of distilled water were added. After mixing, the absorbance of the solution was measured at 560 nm. 0.1 mL of different concentration of the PPE solution was incubated with 0.5 mL of substrate buffer solution at 37 °C for 5 min. Then the same procedure was adopted as above. The inhibitory activity (%) was calculated by using the following formula: % Inhibitory activity = $(1-A/B) \times 100$; Where A is the activity of the enzyme with PPE and B is the activity of enzyme solution without PPE.

2.6. Mouse α -glucosidase Inhibitory Activity

In order to investigate the inhibitory of PPE, an *in vitro* α -glucosidase inhibition test was performed. α -glucosidase from yeast is used extensively as a screening material for α -glucosidase inhibitors, but the results do not always agree with that obtained in mammals. Therefore, we used the mouse small intestine homogenate as an α -glucosidase solution because we speculated that it would better reflect the *in vivo* state. The inhibitory effect was measured using the method slightly modified from a previously described method [17]. After fasting for 20 h, the small intestine between the part immediately below duodenum and the part immediately above the cecum was cut, rinsed with ice-cold saline, and homogenized with 12 mL of maleate buffer (100 mM, pH 6.0). The homogenate was used as the α -glucosidase solution.

The assay mixture consisted of 100 mM maleate buffer (pH 6.0), 500 mM each sugar substrate (maltose and

sucrose) solution (100 μ L), and different concentrations of PPE. It was preincubated for 5 min at 37° C, and the reaction was initiated by adding the crude α -glucosidase solution (50 μ L) to it, followed by incubation for 10 min at 37°C. The glucose released into the reaction mixture was determined with the kit described above. The rate of carbohydrate decomposition was calculated as percentage ratio to the amount of glucose obtained when the carbohydrate was completely digested. The rate of prevention was calculated by the following formula: Inhibition rate (%) = (Amount of glucose produced by the positive control) – (Amount of glucose produced by the addition of PPE) – (Glucose production value in the blank)/ (Amount of glucose produced by the positive control) $\times$ 100.

2.7. Statistical Analysis

The experimental data were expressed as mean $\pm$ SEM. The significance of difference among the various treated groups and control group were analyzed by means of one-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad Instat Software version 3.06 (San Diego, USA). $P < 0.05$ was considered as statistically significant. IC_{50} (inhibitor concentration which produces 50 % inhibition) were estimated using linear regression method of plots of the percent inhibitory activity against the concentration of the tested compounds using Microsoft Excel Software Programme (Microsoft Office 2010 version).

3. Results and Discussion

The aim of the study was to show the different reactivity of porcine pancreatic amylase and rat intestinal α -glucosidase with different concentrations of polyphenolic extract (PPE). As shown in Fig. 1 incubation of PPE at different concentrations with pancreatic α - amylase enzyme caused a dose-dependent decrease in its activity with 50 % inhibition (IC_{50}) at 42.91 μ g/ml for the DNS method. Furthermore, the inhibitory activity of PPE against porcine pancreatic amylase was determined *in vitro* by CNP-G₃ and starch iodine method (Fig. 1). These results show the anti-amylase activity of PPE equivalent phenolic basis, notably for the higher dose tested, the α -amylase

inhibitory activity was relatively high compared to other tested doses. Since PPE was found to inhibit *in vitro*, its activity was then studied using α - amylase in rat plasma *in vivo*. The results were depicted in Fig. 2. An increase in grade concentrations of the PPE and nojirimycin were found to inhibit rat plasma α - amylase activity with 50 % inhibition (IC_{50}) at 54.34 μ g/ml for and 123.41 μ g/ml, respectively. PPE exhibited α -glucosidase inhibitory (maltase and sucrase) activities in a dose-dependent manner (Fig 3). However, the inhibitory effect of PPE was weaker than that of acarbose, which is a well-known α -glucosidase inhibitor.

The onset of Type II diabetes and its associated long-term cardiovascular complications has been linked to persistent postprandial hyperglycemia [18-19]. A number of synthetic drugs available for the treatment of Type II diabetes mellitus and mainly work to affect insulin resistance or defective insulin secretion. However, the use of many of these drugs has been associated with side effects, leading researchers to seek safe and natural sources for new therapeutics [20]. Currently, much focus has been based on the dietary sources of α -amylase and/or α -glucosidase inhibitors to delay the intestinal absorption of digested carbohydrates [21-22]. Alpha-amylase and α -glucosidase is also drug design targets for the development of compounds for the treatment of diabetes, obesity, and hyperlipidemia [23].

Here we report the ability of a phenolic enriched extract of *I. frutescens* to inhibit α - amylase, and α - glucosidase. Chemical analysis showed that the major constituents of PPE are flavonoids and simple phenolic compounds. Our results revealed that the PPE was able to inhibit α -amylase and α -glucosidase activities *in vitro* in a dose-dependent manner. The different extent of α - amylase inhibitory effects of a polyphenolic extract of *I. frutescens* were studied by four different methods. The total phenolic contents seemed to be inversely associated with the amylase inhibition. We speculate that Phenolic flavonoids synergies may be involved in the leaves of *I. frutescens* extract enzyme inhibition mechanism. It has been recently reported that tea polyphenols inhibited glucose transporter of small intestine epithelial cells [24]. In addition, polyphenols controlled the rise in blood glucose level when humans fed with a fixed amount of carbohydrates with food because a negative correlation was indicated by the polyphenolic content and glycemic index [25].

Figure 1. *In vitro* inhibitory effect of polyphenolic extract of *I. frutescens* on α - amylase enzyme using starch iodine, CNP-G3 and DNS method. Values are expressed as mean $\pm$ SEM of triplicate experiments.

Figure 2. *In vitro* inhibitory effect of polyphenolic extract of *I. frutescens* on rat α - amylase enzyme. Values are expressed as mean $\pm$ SEM of triplicate experiments.

Figure 3. The α -glucosidase inhibitory activity of polyphenolic extract and acarbose. Values are expressed as mean $\pm$ S.E.M of three experiments. Inhibitory concentration

The enzyme inhibitors impede digestion through their action of digestive enzymes (amylase and glucosidase) which play a key role in the digestion of plant starch. Our results showed moderate inhibition of α -amylase activity. Inhibitory activities of PPE against α -amylase that our results confirmed suggest its potential in prevention and therapy of obesity and diabetes. Living organisms use enzyme inhibitors as a major tool to regulate glycolytic activities of α -amylase. In most of the cases, the mechanism of inhibition occurs through the direct blockage of the active centre at several subsites of the enzyme [26].

Extract and/or purified inhibitors may be used as a drug design target for the treatment of diabetes by exploring their potential pharmacological effects. It is widely recommended that a wide and deep investigation of therapeutic enzyme inhibitors is needed to explore the medicinal values of Indian medicinal plants used ethno-medically for treating diabetes. We hypothesize that the health benefits of *I. frutescens* against diabetes mellitus may be due to the α -amylase and α -glucosidase inhibitory activities of the apigenin, luteolin, quercetin and other polyphenolics compounds, which are found within leaves of *I. frutescens* has been shown in other studies [27]. The *in vitro* effectiveness of luteolin, quercetin and apigenin on the inhibition of α -glucosidase and α -amylase have been previously described [28-29]. These compounds may play an important role in controlling dietary glucose uptake in the small intestine by PPE extract, due to its α -amylase, and α -glucosidase inhibitory activities.

From the chemical structures and inhibitory activity

relationship, poly-hydroxyl groups in the flavonoids seem to play an important role in the inhibition of these enzymes. Since many plant polyphenols are known to form a hydrophobic bond to proteins and would affect the functions of not only α -glucosidase but also the Na^+ -glucose transporter and the Na^+ -dependent glucose transporter protein as has been shown in the small intestinal epithelium of mice [30]. Food grade phenolic α -amylase and α -glucosidase enzyme inhibitors from herbal extracts are potentially safer, and therefore may be proffered alternative for inhibition of carbohydrate breakdown and control of glycemic index of food products. It has been reported that α -glucosidase inhibitors usually do not alter the total amount of carbohydrate absorbed and therefore do not cause any net nutritional caloric loss although slow down carbohydrate digestion. Our studies suggest a new biological activity of this plant as it may mildly inhibit α -amylase and α -glucosidase activities, consequently, this may be expected to clinically contribute to the gentle improvement of postprandial hyperglycemia in diabetic patients. Further research is aimed at investigating the potential role of α -amylase and α -glucosidase inhibitors present in the polyphenolic extract of *I. chnocarpos frutescens*.

4. Conclusions

In conclusion, the outcomes of the current investigation confirm that the polyphenolic extract of *I. chnocarpos frutescens* is effective against α -amylase and α -glucosidase,

which may be useful to decrease the postprandial glucose levels. Isolation of the active phytochemicals is presently being pursued in our research laboratory to better understand the mechanism of this enzyme inhibition. Furthermore, our results support the ethnomedical use of plants in medicine based on their inhibitory activity of carbohydrate absorption from the gut. Only a thorough and full-fledged study can rationalize the use in humans.

List of Abbreviations

PPE= Polyphenol extract
 CNPG3=2-chloro-p-nitrophenyl- α -D-maltotrioxide
 DNS=dinitrosalicylic acid KSCN = Potassium thiocyanate
 Abs= Absorbance
 CNP= 2-chloro-4-nitrophenol
 CPCSEA=Committee for the Purpose of Control and Supervision of Experiments on Animals
 ANOVA= Analysis of variance

Conflicts of Interest

None

Author's Contribution

KC designed the study, performed the experiments, did the literature searches, and wrote the manuscript. ET and SRD managed the experimental process and provided technical assistance. Author SCM, RS and VK contributed to the protocol and provided general supervision of the study. All authors have read and approved the submission of the manuscript.

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Declarations

Experiments were performed complied with the rulings of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi, India and the study was permitted by the institutional ethical committee of the Jadavpur University.

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