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Phytochemical Analysis and Inhibitory Activity of Aqueous & Methanol Extract of *Syzygium Cumini* and *Stevia Rebaudiana* on Porcine Pancreatic Amylase

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Abstract – Diabetes has become a common global health problem affecting >170 million people worldwide. It is one of the leading causes of death, estimated number to be 366 million by year 2030. The majority of diabetes (~90%) is of type II diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver. An in vitro anti-diabetic activity of two indigenous medicinal plants were evaluated by α-amylase inhibition assay using the methanol and water extracts. Qualitative phytochemical analysis was performed to evaluate the presence of flavonoids, alkaloids, glycosides, saponins, carbohydrates and tannins. Traditional Medicines obtained from medicinal plants are used by about 40-60% of the world's population. Though there are many approaches to control diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost. In this study, the inhibitory effect of Syzygium cumini seeds and Stevia rebaudiana leaves extract on porcine pancreatic amylase was evaluted. The aqueous extracts of Syzygium cumini seeds significantly exhibited higher (p < 0.05) inhibition of α-amylase activity than Stevia rebaudiana leaves as compared to standard drug acarbose.

Keywords – α-Amylase, Diabetes, Inhibition, Syzygium Cumini and Stevia Rebaudiana

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1. INTRODUCTION

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and postprandial blood sugar levels. The global prevalence of diabetes is estimated to increase by 5.4% by the year 2025. WHO has predicted that the major burden will occur in developing countries. Studies conducted in India in the last decade have highlighted that not only is the prevalence of diabetes high but also that it is increasing rapidly in the urban population [1]. Type I diabetes (insulin dependent) is caused due to insulin insufficiency because of lack of functional beta cells. Though patho physiology of diabetes remains to be fully understood, experimental evidences suggest the involvement of free radicals in the pathogenesis of diabetes and more importantly in the development of diabetic complications[12,13,16]. Free radicals are capable of damaging cellular molecules, DNA, proteins and lipids leading to altered cellular functions. Many recent studies reveal that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models [5], as well as reducing the severity of diabetic complications [12]. For the development of diabetic complications, the abnormalities produced in lipids and proteins are the major etiologic factors. In diabetic patients, extra- cellular and long lived proteins, such as elastin, laminin, collagen are the major targets of free radicals. These proteins are modified to form glycoproteins due to hyperglycemia. [5]. During diabetes, lipoproteins are oxidized by free radicals. There are also multiple abnormalities of lipoprotein metabolism in very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) in diabetes. Lipid peroxidation is enhanced due to increased oxidative stress in diabetic condition. Apart from this, advanced glycation end products (AGEs) are formed by non-enzymatic glycosylation of proteins. AGEs tend to accumulate on long-lived molecules in tissues and generate abnormalities in cell and tissue functions [4, 15]. To manage post-prandial hyper-glycaemia at digestive level, glucosidase inhibitors such as Acarbose, miglitol and voglibose are used. Although several therapies are in use for treatment, there are certain limitations due to high cost and side effects such as development of hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity etc., [14]. Based on recent advances and the involvement of oxidative stress in complicating diabetes mellitus, efforts are on to find suitable

antidiabetic and antioxidant therapy. It has been shown that the activity of the HPA (human pancreatic α-amylase) in the small intestine correlates to an increase in post- prandial glucose levels, the control of which is therefore an important aspect in the treatment of type 2 diabetes. Herbal medicines are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents. Indigenous medicinal plants have been identified and used effectively to maintain normal blood glucose level for a long period of time. Different mechanisms of actions that control the normal blood glucose level mimics insulin activity, reduction of insulin resistance and inhibition of α-amylase enzymes activity is of importance [6]. α-amylase as an inhibitor can effectively reduce the glucose absorption, by blocking the breakdown of long-chain carbohydrates into glucose. Inhibition of the enzyme activity has become the vital therapeutic approach to retard glucose absorption and suppress postprandial hyperglycemia [3]. Parenteral administration of insulin and oral use of hypoglycemic drugs like sulphonylureas, biguanides, D-phenylalanine derivatives, and meglitinides inhibitors is the routine approach for the treatment of diabetes. Prolonged use of these allopathic drugs is associated with side effects like nausea, gastrointestinal disturbance, headache, dizziness, lactic acidosis, pernicious anemia, dyspepsia and joint pain [16]. Herbal drugs are economic and possesses no or less adverse effects as compared to anti-hyperglycemic drugs [11]. Over 400 traditional plant treatments for diabetes have been documented, although only a small number of these have received scientific and medical evaluation to assess their efficacy. Many medicinal plants are being consumed or used in Ayurveda for the treatment of diabetes [9]. A number of medicinal plants and their formulations are used for treating diabetes in the traditional Indian Ayurvedic system as well as in ethnomedicinal practices. Pharmacological properties of α-glucosidase inhibitors such as acarbose that can also inhibit pancreatic α-amylase revealed that the complications of DM such as onset of renal, retinal, lens and neurological changes and the development of ischaemic myocardial lesions are prevented or delayed. Long-term day-to- day management of diabetes, with acarbose is well tolerated and can improve glycaemic control as monotherapy, as well as in combination therapy *Syzygium cumini* and *Stevia rebaudiana* are well known in Ayurveda to possess anti-diabetic properties. Structurally as well as mechanistically, PPA (Porcine pancreatic α-amylase) is closely related to the HPA (Human pancreatic α-amylase). Hence, sequential solvent extracts of the above medicinal plants were screened for the presence of PPA inhibitors, Pancreatic α- amylase, is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to maltose and finally to glucose. Degradation of this dietary starch proceeds rapidly and leads to elevated post prandial hyperglycemia (PPHG). Hence retardation of starch digestion by inhibition of enzymes such as α-amylase would play a key role in the control of diabetes. Inhibitors such as acarbose, miglitol, and voglibose, are known to inhibit a wide range of glycosidases such as α- glucosidase and α-amylase. A large number of medicinal plants are growing surrounding Shivamogga district of Karnataka in India, and many of them might have an anti-diabetic potential. Hence, the present research work was carried out to explore *in vitro* anti-diabetic activity or inhibitory effect of two medicinal plant (*Syzygium cumini* and *Stevia rebaudiana*) extracts on PPA.

2. MATERIALS AND METHODS

All the chemicals procured for this work were of analytical grade, (PPA), Starch, chloroform, methanol, iso-propanol, methyl-butyl tertiary ether, acetone, and dimethylsulfoxide (DMSO) were procured from S.D. Fine Chem. Ltd, Mumbai, India. Porcine pancreatic α-amylase, 3,5-dinitrosalicylicacid (DNSA) was obtained from HiMedia Laboratories, Mumbai, India. Human pancreatic α-amylase (HPA) and acarbose were purchased from Sigma Aldrich, Bangalore.

2.1 Collection and processing of plant material

The two plant materials were collected from surrounding regions of Shivamogga district, Karnataka (India). Collected plant materials were identified and authenticated by Dr. T. Parmeshwar Naik, a renowned botanist from Sahyadri Science College, Shivamogga. The collected plant materials were washed with tap water followed by shade drying. The dried material was grinded to fine powder and stored in an air-tight container for further use.

2.2 Preparation of Plant Extracts

The plant materials were subjected to air drying, 60–100 gm of the plant material was macerated with liquid nitrogen, and extracted in polar to nonpolar solvent on an increasing degree of non- polarity. Different extracts were sequentially obtained with cold water, hot water, methanol, iso-propanol, chloroform, acetone, and methyl-butyl tertiary ether. The extraction procedures were carried out taking into consideration the fact that traditional methods of preparing herbal formulations are mainly aqueous. Also, aqueous extracts contain peptides, proteins, or glycans, which would otherwise be denatured by organic solvents and at high-temperature extraction. Distilled water was added to the crushed material in a ratio of 1: 4 (w/v) and kept at 30°C (24 hrs) and 55°C (2 hrs) at 120 RPM for cold-and hot-water extracts, respectively. For each solvent, the extract was filtered, centrifuged, and the residue collected for subsequent solvent extraction. The organic solvents were added in a ratio of 1: 3 (w/v) and refluxed with the residue for 3 hrs at their respective boiling temperatures. Each extract was filtered and stored at −20°C for future work.

2.3 Phytochemical screening

Qualitative phytochemical screening was performed for each extract as per standard procedures described by Harborne [8].

2.3.1 Tests for alkaloid

Test solution was prepared by tritrating 40 to 50 mg extract with dilute acid (10 % acetic acid or 1 to 5 % hydrochloric acid). After filtration, 0.5 to 1 mL filtrate was added with 1 to 2 mL of following reagents.

Mayer's test

Mayer's reagent (Solution I: Dissolve 1.36 g $HgCl_2$ in 60 mL water; Solution II: Dissolve 5 g potassium iodide in 10 mL water. Combine these two solutions and add water up to 100 mL). Test solution (0.5 to 1 mL) was added with 1 to 2 mL of Mayer's reagent and development of white or buff colour precipitates indicates the presence of alkaloid.

2.3.2 Test for flavonoid

Test solution was prepared by dissolving 50 to 100 mg extract in 10 mL methanol/water.

Shinoda test

Test solution (1 to 2 mL) was added with a pinch of magnesium metal powder and a few drops of concentrated hydrochloric acid. Development of orange, pink, red to purple colours indicated the presence of flavones, flavonols or xanthones.

Sulfuric acid test

Test solution (1 to 2 mL) was added with few drops of concentrated sulfuric acid from the side wall of test tubes. Flavones and flavonols dissolve into concentrated H_2SO_4, producing a deep yellow coloured solution. Flavanones give orange to red colour.

2.3.3 Test for saponin

Take 0.1 to 0.2 g of extract and add 10 mL distilled water, and shake vigorously. The appearance of froth that stabilizes for 10 to 15 minutes indicates the presence of saponin.

2.3.4 Test for glycoside

Salkowaski's test

To the crude extract (about 50 to 100 mg) taken add 2 mL of chloroform, shake well and then add 2 mL of concentrated H_2SO_4 along the side of the test tube. The development of reddish brown colour at the interface indicates the presence of sterol.

2.3.5 Test for sugars

Molisch's Test

Molisch's reagent (Dissolve 1 g of α- naphthol in 10 mL of methanol or isopropyl alcohol). Test solution (1 to 2 mL) was mixed in a test tube containing 0.5 mL of water, and added with two drops of Molisch's reagent followed by 1 mL of concentrated sulphuric acid from the side of the inclined test tube. Appearance of red brown/violet ring at the interface of acid and aqueous solution indicates the presence of sugars.

2.3.6 Test for tannins

Ferric Chloride Test

Prepare 5 % solution of ferric chloride in 90 % methanol. Test solution (1 to 2 mL) was added with few drops of ferric chloride solution and development of dark green or deep blue colour indicates the presence of tannins.

2.4. α-Amylase Inhibition Assay

Initially Porcine Pancreatic Amylase was used for preliminary screening of α-amylase inhibitors from the extracts obtained. Chromogenic DNSA method was employed to study the inhibition property [17]. The total assay mixture composed of 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 0.04 units of PPA solution, and plant extracts at concentration in the range of 0.1–1.5 mg mL−1 (w/v), incubated at 37°C for 10 min. After pre-incubation, 500 µL of 1% (v/v) starch solution in the above buffer was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 mL DNSA reagent, placed in boiling water bath for 5 min, cooled to room temperature, diluted, and the absorbance was measured at 540 nm. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls were included. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per min under the assay conditions. For the determination of the inhibitor concentration at which 50% inhibition of enzyme activity occurs (IC_{50}), the assay was performed as above except that the inhibitor/plant extract concentrations varied from 0.1–150 µg. Acarbose was used as a positive control in a concentration range of 6.5µg–32.6 µg. The IC_{50} values were determined from plots of percent inhibition versus log inhibitor concentration and calculated by logarithmic regression analysis from the mean inhibitory values. The IC_{50} values were defined as the concentration of the extracts, containing the α-amylase inhibitor that inhibited 50% of the PPA.

% Relative enzyme activity = (enzyme activity of test/enzyme activity of control) * 100

% inhibition in the α-amylase activity = (100 −% relative enzyme activity).

3. RESULTS & DISCUSSION

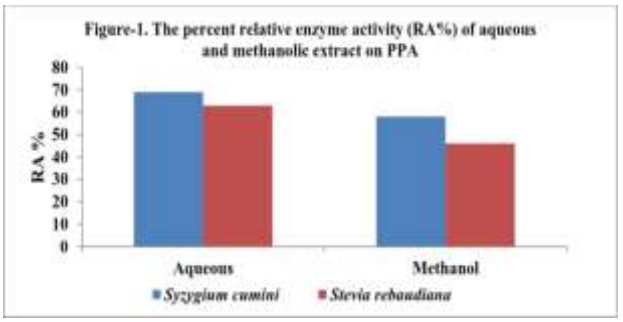
Qualitative analysis of different phytochemical compounds of the two plant extracts obtained from Shivamogga region in Karnataka (Table-1)**.** Extract of *Syzygium cumini* and *Stevia rebaudiana* has shown the presence of various phytochemical ingredients like flavonoids, alkaloids, tannins and glycosides which might be responsible for *in vitro* anti-diabetic effects. Early reports suggest that plants containing natural antioxidants like tannins, flavonoids Vitamin C and Vitamin E can inhibit the lipid peroxidation and generation of diabetes-induced reactive oxygen species (ROS), and prevent the further destruction of β-cells of pancreas [10]. Of the different extracts of two medicinal plants obtained from Shivamogga region in Karnataka, the aqueous extract of *Syzygium cumini* have shown good inhibitory activity against PPA (α-amylase), when sequentially extracted with polar to non- polar solvents. Commonly its always the cold-and hot-water extracts used in the traditional method of preparing medicines in Ayurveda, and there is high possibility of missing out on bioactive principles with better amylase inhibitory potential from less polar solvents. Per cent inhibitions of α-amylase enzymatic activity is shown respectively (Table-2). An α-amylase is responsible for the conversion of starch into mono and disaccharides which ultimately increases the blood sugar level. PPA was used as a target enzyme for screening the inhibitory activity from the above mentioned plant extracts. The acqueous extracts of *Syzygium cumini* seeds 100 µg/mL have shown good per cent inhibition of PPA (α-amylase activity of 53.09 ± 0.50 %) than the extracts of *Stevia rebaudiana.* The control reaction representing 100% enzyme activity was 0.20 µ/mL for PPA. The dried extract samples dissolved in DMSO with a final yield of 1.5 mg mL−1, didn't affect the enzyme activity of PPA at that concentration used. The appropriately diluted plant extract was used for enzyme inhibition assay and the activity obtained with each extract was normalized to percent relative activity (Figure-1) from which the percent inhibition was calculated. The most significant inhibitory activity was obtained with the aqueous extracts of, *Syzygium cumini* than *Stevia rebaudiana.* The known PPA inhibitor, acarbose, taken as a positive control exhibited an IC_{50} value of 79.86 µgml (Table- 2). The presence of bioactive compounds like alkaloids, amino acids, flavonoids, glycosides, saponins, steroids, tannins triterpenoids and mycaminose in *Syzygium cumini* may be responsible for the inhibitory activities [2] than the extracts of *Stevia rebaudiana* containing bioactive compound (Stevioside) have shown quite lower activity against PPA [7].

Table-1. Phyto-chemical screening of the plant extracts

PLANT SPECIES	FAMILY	PART USED	EXTRACT	PHYTOCHEMICALS PRESENT: Alkaloids	Sugars	Saponins	Glycosides	Flavanoids	Tannins
Syzygium cumini	Myrtaceae	Seeds	Cold water	+	-	+	-	+	-
			Hot water	-	-	+	+	-	+
			Methanol	+	-	+	+	-	+
			Isopropanol	-	-	-	-	+	+
			Chloroform	+	-	+	+	+	+
			Acetone	-	-	-	-	-	-
			Methyl-butyl	-	-	+	-	-	-
Stevia rebaudiana	Asteraceae	Leaves	Cold water	-	-	-	-	-	-
			Hot water	-	-	+	-	-	+
			Methanol	+	+	-	+	-	+
			Isopropanol	+	+	-	-	+	+
			Chloroform	+	+	-	-	-	+
			Acetone	-	-	-	+	-	+
			Methyl-butyl tertiary ether	+	-	-	-	-	-

Table-2: IC_{50} OF ACARBOSE

Standard drug	Concentrations (µg/ml)	% inhibition	Ic_{50} value
ACARBOSE	100	57.48	79.86
	80	46.86	
	60	37.93	
	40	26.64	
	20	20.41	
	10	17.10	



4. CONCLUSION

Most of the Indian population from the rural side utilize these plants for food purposes as well to treat diabetes. The leaves, seeds and bark of *Syzygium cumini* and leaf extracts *Stevia rebaudiana* contain active phytochemicals viz. flavonoids, alkaloids, glycosides, saponins and tannins. Our results indicate that retardation of starch hydrolysis by inhibition of PPA activity of these plant extracts leads to a reduction in glucose concentrations, significantly exhibiting *in vitro* anti-diabetic activity. *Syzygium cumini* exhibited significant inhibition of PPA than *Stevia rebaudiana*, suggesting that they contain compounds capable of PPA inhibition. Preliminary phytochemical analysis of the leaf extracts suggests the occurrence of proteins/peptides and polyphenols in cold-and hot-water extracts while the tannins, alkaloids, flavonoids, and saponins are found in non-polar extracts. Flavonoids and polyphenolics may be responsible for hypoglycemic activity. In conclusion, these natural plant metabolites might have therapeutic potential for the control of postprandial blood glucose levels and development of novel and effective anti-diabetic drugs in future.

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