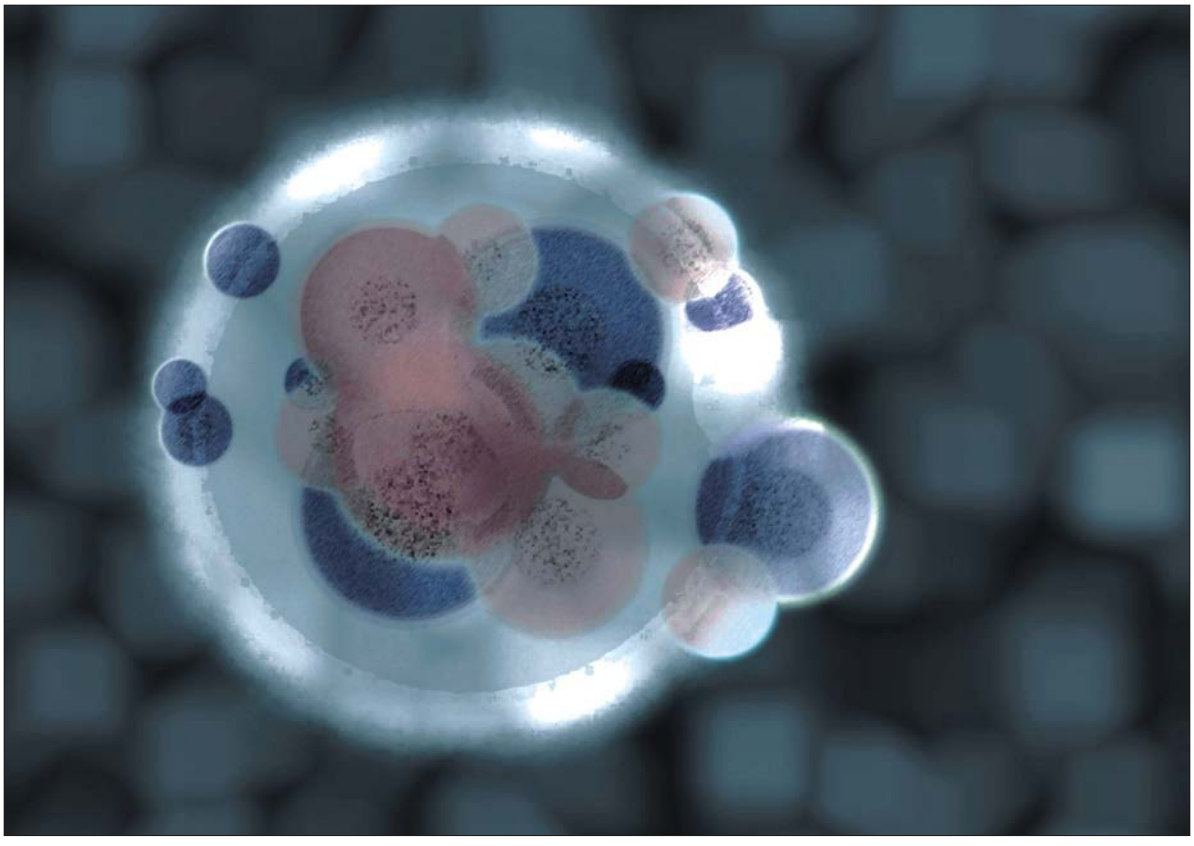

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Enzymatic *in vitro* Anti-diabetic Activity of Few Traditional Indian Medicinal Plants

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Abstract: Controlling post-prandial hyperglycaemia through enzymatic inhibition of starch degradation is an effective therapeutic approach in the management of diabetes mellitus. To achieve this, twelve indigenous antidiabetic Indian medicinal plants such as *Trigonella foenum-graecum, Ocimum sanctum, Aegle marmelos, Plantago ovata, Catharanthus roseus, Alium cepa, Azadirachta indica, Aloe vera, Magnifera indica, Terminalia chebula, Eugenia jambolana* and *Linum usitatisumum* were subjected to sequential solvent extraction and thereafter, 48 fractions were screened for their α-amylase inhibitory potential at three dosage levels *in vitro*. Out of the 144 samples, *Eugenia jambolana* water extract showed maximum α-amylase inhibitory activity with IC_{50} value 1.33 mg mL^{-1} in comparison with standard drug acarbose (IC_{50} value 0.86 mg mL^{-1}). Quantitative phytochemical analysis of the lead extract revealed the presence of phenolic content as 69.68 mg tannic acid equivalent g^{-1} while flavonoidal content as 57.39 mg rutin equivalent g^{-1}. Present study indicated *Eugenia jambolana* as a potential α-amylase inhibitor in the management of diabetes.

Key words: α-amylase inhibitory activity, diabetes mellitus, postprandial hyperglycaemia

INTRODUCTION

Diabetes is a metabolic disorder associated with multiple complications and premature mortality, accounting for a major chunk total health care expenditure in many countries (King *et al*., 1998). If not treated properly, this leads to long-term damage of various organs mainly retinopathy, cardiovascular disorders, nephropathy and neuropathy. The main symptoms of this disease are thirst, polyuria, blurring of vision and weight loss (WHO, 1999).

The treatment of diabetes mellitus primarily aims at achieving effective control of the elevated blood sugar levels. The available therapies for the control of diabetes mainly target stimulation or enhancement of action of insulin at the target tissues through oral hypoglycaemic agents and inhibition of degradation of dietary starch by carbohydrate hydrolysing enzymes such as α-amylase and α-glucosidases to decrease the post-prandial hyperglycaemia.

Controlling post-prandial hyperglycaemia by retarding the absorption of glucose through the inhibition of the two main enzymes i.e. α-amylase and α-glucosidase in the digestive tract. This action delays carbohydrate digestion, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Rhabasa-Lhoret and Chiasson, 2004). Examples of such inhibitors are acarbose, miglitol and voglibose which are currently been used in the market (Bailey, 2003). It is well established that insulin and other oral anti-diabetic agents like biguanids, sulfonylureas and thiozolidinediones are known to control hyperglycaemia but none of them are free from side effects (Valiathan, 1998). Therefore, search for effective antidiabetic agents with minimal side effects is warranted and this can be accomplished probably from plants (Ray *et al*., 2010). Since the plants selected for this study are very well known for their antidiabteic potential in the folklore medicines in India but none has been screened for their α-amylase inhibitory activity so far. On this ground, the present study was planned to investigate twelve indigenous medicinal plants such as *Trigonella foenum-graecum, Ocimum sactum, Aegle marmelos, Plantago ovata, Catharanthus roseus, Alium cepa, Azadirachta indica, Aloe vera, Mangifera indica, Terminalia chebula, Eugenia jambolana, Linum usitatisumum* for their *in vitro* α-amylase inhibitory potential.

MATERIALS AND METHODS

Chemicals: Porcine pancreatic α-amylase enzyme, DNSA (3,5-dinitrosalicylic acid), DMSO (dimethyl sulphoxide), sodium potassium tartrate, sodium hydroxide were purchased from SRL Pvt. Ltd, Mumbai, India. Potato starch, disodium hydrogen phosphate, potassium dihydrogen phosphate, methanol, petroleum ether, ethyl acetate, aluminium trichloride, potassium acetate, sodium carbonate, tannic acid were purchased from Loba chemie,

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Mumbai, India. Chloroform, Folin-Ciocalteau reagent were purchased from CDH chemicals, New Delhi, India. Rutin was purchased from Utan biotech. Ltd, Rajasthan, India. Acarbose was purchased from Sigma Aldrich, USA.

Plant materials: The traditional Indian medicinal plants mentioned in Table 1, were collected from herbal garden of Maharshi Dayanand University, Rohtak, Haryana and local market of Khari baoli, Delhi (India). The plant material was properly identified and Voucher specimen were kept in the department for future reference.

Extraction and fractionation: All selected plant parts were dried at room temperature for 25 days. The dried plant parts were finely crushed, powdered and extracted with methanol using soxhlet apparatus. The extracts were filtered and concentrated using rotary evaporator. The percentage yields of each dried methanol extracts were calculated. The methanol extracts of plants were suspended in water and partitioned successively with petroleum-ether, chloroform and ethyl acetate to get the, respective extracts. Remaining aqueous final fraction kept as such. Petroleum-ether, chloroform and ethyl acetate extracts were concentrated using rotary evaporator while the aqueous extracts were lyophilized. Each dried extracts were stored in refrigerator for future use and dissolved in DMSO to give different concentrations for estimation of *in vitro* α-amylase inhibitory activity.

α-amylase inhibition test: The α-amylase inhibitory activity for each extract was determined based on the colorimetric method described by Nickavar *et al.* (2008). Briefly, the starch solution was obtained by stirring and boiling 0.25 g of soluble potato starch in 50 mL of 20 mM phosphate buffer for 15 min. The enzyme solution was prepared by mixing 1 mg of porcine pancreatic α-amylase in 100 mL of 20 mM phosphate buffer (pH 6.9). The extracts were dissolved in DMSO to give different concentrations. The colour reagent was a solution containing 96 mM 3,5-dinitrosalicylic acid (20 mL), 5.31 M sodium potassium tartrate in 2 M sodium hydroxide (8 mL) and deionized water (12 mL).

One mililiter of each plant extract and one mL enzyme solution were mixed in a tube and incubated at 25°C for 10 min. To 1 mL of this mixture was added 1 mL of starch solution and the tube incubated at 25°C for 10 min. Then, 1 mL of colour reagent was added and the closed tube placed into water bath at 85°C. After 15 min, the reaction mixture was removed from the water bath, cooled and diluted with 9 mL distilled water and the absorbance value determined at 540 nm in spectrophotometer. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. Individual blanks were prepared for correcting the background absorbance. The other procedures were carried out as above. Control was conducted in an identical manner replacing the plant extracts with 1 mL DMSO. Acarbose solution was used as positive control.

The inhibition percentage of α-amylase was calculated using following formula:

$$I\alpha\text{-amylase}\,\% = 100 \times \frac{(\Delta A_{Control} - \Delta A_{Sample})}{\Delta A_{Control}}$$

Where:

$\Delta A_{Control} = A_{Test} - A_{Blank}$

$\Delta A_{Sample} = A_{Test} - A_{Blank}$

Table 1: List of the medicinal plants screened for α- amylase inhibitory activity

Plants name	Family	Parts used	Hypoglycemic and medicinal properties
Trigonella foenum-graecum	Leguminosae	Seeds	Hypoglycemic activity due to presence of trigonellin and 4-hydroxy isoleucine, Decreases post prandial blood glucose level.
Ocimum sanctum	Labiatae	Leaves	Lowered blood sugar level, potent hypoglycemic and hypolipidemic effect
Aegle marmelos	Rutaceae	Leaves	Anti-hyperglycemic activity along with decreased cholesterol and blood urea, increased plasma insulin level
Plantago ovata	Plantaginaceae	Seeds	Treatment of hyperglycaemia as well as dyslipidaemia in type 2 diabetes
Catharanthus roseus	Apocynaceae	Leaves	Reduces blood glucose by enhancing secretion of insulin from α-cells of Langerhans or through extra pancreatic mechanism
Alium cepa	Liliaceae	Bulbs	Lowered blood sugar level, antioxidant and hypoglycemic activity
Azadirachta indica	Meliaceae	Leaves	Lowered blood sugar level, hypoglycemic activity
Aloe vera	Liliaceae	Juice	Improved the plasma insulin level, decreases fasting glucose levels, hepatic transaminases, plasma and liver cholesterol, triglycerides, free fatty acids and phospholipids.
Magnifera indica	Anacardiaceae	Leaves	Reduces glucose absorption in type 2 diabetes. Stimulates glycogenesis in liver causing reduction in blood glucose level.
Terminalia chebula	Combretaceae	Fruits	Reduced the levels of blood glucose and serum lipids, Inhibits advanced glycosylation end products, which contribute to renal damage.
Eugenia jambolana	Myrtaceae	Leaves	Hypoglycemic activity along with insulin released from ß-cells, regulated blood sugar level
Linum usitatisumum	Linaceae	Seeds	Reduces fasting blood sugar levels, total cholesterol; reduces carbo- hydrate absorption from gut and clinical symptoms of diabetes associated with dyslipidemia.

Phytochemical analysis

Preliminary phytochemical analysis: Lead extracts positive for α-amylase inhibition were tested for the presence or absences of various phytochemicals in accordance to the standard chemical tests mentioned in standard book (Trease and Evans, 1989).

Determination of Total phenolic content: The total phenolic content of the extracts were determined by the Folin-Ciocalteau method. 5 gram per 50 mL of sample was filtered with whatman paper. 0.5 mL of the sample was added to 2.5 mL of 0.2 N Folin-Ciocalteau reagent and placed for 5 min. 2 mL of 75 g L^{-1} of Na_2CO_3 were then added and the total volume made up to 25 mL using distilled water. The above solution was then kept for incubation at room temperature for 2 h. Absorbance was measured at 760 nm using spectrophotometer. Tannic acid (0-600 mg mL^{-1}) was used to produce standard calibration curve. The total phenolic content was expressed in mg of Tannic Acid Equivalents (TAE) g^{-1} of extract (Subramanian *et al*., 2008).

Determination of total flavonoid content: The total flavonoid content was determined according to the aluminium chloride colorimetric method. Each plant extracts (2 mL, 0.3 mg mL^{-1}) in methanol were mixed with 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. After the 40 min incubation at the room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm. Rutin was chosen as a standard the concentration range (0.005 to 0.1 mg mL^{-1}) and the total flavonoid content was expressed as milligram RE per g of dry extracts (Stanojevic *et al*., 2009).

Statistical analysis: All experiments were performed in triplicate and the data were expressed as mean±SEM (standard error of the mean). Linear regression was performed for calculating inhibitory concentration 50% (IC50). Microsoft EXCEL program and graph pad instat 3.0 software was used for data analysis. One-way analysis of variance (ANOVA) followed by post hoc Dunnet's t-test was used to assess the presence of significant differences ($p<0.01$).

RESULTS AND DISCUSSION

Extracts with α-amylase inhibitory activity: Out of 144 samples screened for α-amylase inhibitory activity, 8 plant fractions were considered to have good activity with IC_{50} value ranging from 1.330 to 3.716 mg mL^{-1}. The aqueous extracts of *Azadirachta indica* and *Eugenia jambolana* exhibited 28.15 and 43.12% inhibition at concentration of 1 mg mL^{-1}. Ethyl acetate extracts of *Ocimum sanctum* (25.76%) inhibition followed by *Magnifera indica* (26.33%), *Eugenia jambolana* (37.53%), *Allium cepa* (38.14%) and *Azadirachta indica* (39.23%) at 1 mg mL^{-1} respectively. Significant and strong inhibition was observed for pet ether extracts of *Eugenia jambolana* (29.37%) at 1 mg mL^{-1}. Percent inhibition, IC_{50} value and percent relative enzyme activity is represented in Table 2

Table 2: Extracts with maximum α-amylase inhibitory activity

Plant species	Extract	Conc.(mg mL^{-1})	%Inhibition (Mean±SEM)	% Relative enzyme activity (100% inhibition)	IC_{50} values
Eugenia jambolana	Water	1	43.12±0.33	56.88±0.33	1.330
		2	62.33±0.69	37.67±0.69	
		4	79.41±0.15	20.59±0.15	
Azadirachta indica	Ethyl acetate	1	39.23±0.15	60.77±0.15	1.725
		2	56.11±0.34	43.89±0.34	
		4	77.24±0.63	22.76±0.63	
Allium cepa	Ethyl acetate	1	38.14±0.87	61.86±0.87	1.847
		2	55.12±0.73	44.88±0.73	
		4	73.24±0.52	26.76±0.52	
Azadirachta indica	Water	1	28.15±0.26	71.85±0.26	
		2	46.73±0.43	53.27±0.43	2.571
		4	66.33±0.38	33.67±0.38	
Eugenia jambolana	Petroleum ether	1	29.37±0.18	70.63±0.18	2.727
		2	45.13±0.93	54.87±0.93	
		4	62.74±0.23	37.26±0.23	
Magnifera indica	Ethyl acetate	1	26.33±0.35	73.67±0.35	2.786
		2	47.73±1.03	52.27±1.03	
		4	61.13±0.18	38.87±0.18	
Ocimum sanctum	Ethyl acetate	1	25.76±0.92	74.24±0.92	2.984
		2	43.32±1.13	56.68±1.13	
		4	59.70±0.38	40.30±0.38	
Eugenia jambolana	Ethyl acetate	1	37.53±0.32	62.47±0.32	3.716
		2	42.49±0.78	57.51±0.78	
		4	51.30±0.55	48.70±0.55	

Values are expressed as mean±SEM; (n = 3), SEM: Standard error mean

Table 3: Inhibitory effects of extracts on α-amylase activity

Plant species	Family	Parts used	Petroleum ether	Chloroform	Ethyl acetate	Water
Trigonella foenum-graecum	Leguminosae	Seeds	+	-	+	+
Ocimum sanctum	Labiatae	Leaves	-	-	+++	+
Aegle marmelos	Rutaceae	Leaves	-	-	-	+
Plantago ovata	Plantaginaceae	Seeds	-	-	-	-
Catharanthus roseus	Apocynaceae	Leaves	-	-	-	-
Alium cepa	Liliaceae	Bulbs	-	++	+++	-
Azadirachta indica	Meliaceae	Leaves	-	-	+++	+++
Aloe vera	Liliaceae	Juice	-	-	-	++
Magnifera indica	Anacardiaceae	Leaves	-	-	+++	-
Terminalia chebula	Combretaceae	Fruits	-	-	+	++
Eugenia jambolana	Myrtaceae	Leaves	+++	++	+++	+++
Linum usitatisumum	Linaceae	Seeds	-	-	-	+

+++: extracts with maximum α-amylase inhibitory activity, ++: extracts with moderate α-amylase inhibitory activity, +: extracts with minimum α-amylase inhibitory activity, -: extracts with no α-amylase inhibitory activity

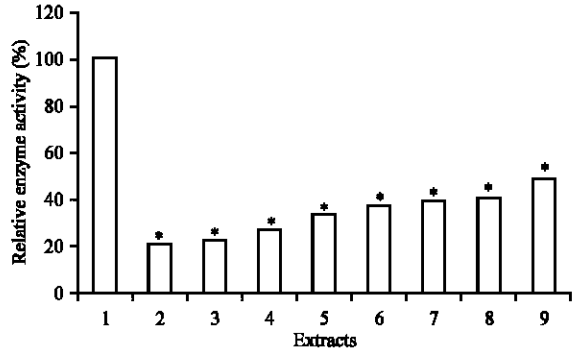

Fig.1: The percentage relative α-amylase enzyme inhibition activity of different extracts. Porcine pancreatic a-amylase served as control. Values are expressed as mean±SEM; (n = 3), 1: Control, 2: E. jambolana aqueous extract, 3: *A. indica* ethyl acetate extract, 4: *A. cepa* ethyl acetate extract, 5: A. indica aqueous extract, 6: *E. jambolana* petroleum ether extract, 7: *M. indica* ethyl acetate extract, 8: *O. sanctum* ethyl acetate extract, 9: *E. jambolana* ethyl acetate extract. One-way analysis of variance (ANOVA) followed by *post hoc* Dunnet's t-test was used and bars with asterisks (*) show significant value ($p<0.01$) with respect to control

and Fig. 1. Four plant fractions i.e. chloroform extracts of *Allium cepa, Eugenia jambolana* and aqueous extracts of *Terminalia chebula, Aloe vera* showed moderate α-amylase inhibitory activity with percentage inhibition ranging from 17.38±0.23 to 48.36±0.47 at different doses. Seven plant fractions i.e. aqueous extracts of *Ocimum sanctum, Trigonella foenum-graecum, Linum usitatisumum, Aegle marmelos,* ethyl acetate extracts of *Trigonella foenum-graecum, Terminalia chebula* and petroleum ether extract of *Trigonella foenum-graecum* showed minimum α-amylase inhibitory activity and rest of the fractions showed no significant α-amylase inhibitory activity (Table 3). Acarbose was taken as a positive control with an IC_{50} value 0.86 mg mL^{-1}.

Phytochemical analysis: Preliminary qualitative phytochemical analysis was performed to determine the probable type of compounds present in the extracts responsible for α- amylase inhibition. The total phenolics content estimated by Folin-Ciocalteau method was found to be 69.68 mg tannic acid equivalent g^{-1} while flavonoidal content as 57.39 mg rutin equivalent g^{-1}.

The above data revealed that phenolic and flavonoidal compounds present in the lead extract of *Eugenia jambolana* may be the responsible components for the observed activity due to their radical scavenging activity as well as α-amylase inhibiting properties (Sawa *et al*., 1999). Reported studies say that these compounds are able to inhibit the activities of digestive enzymes due to their ability to bind with proteins thus contribute to the lowering of postprandial hyperglycaemia (Kim *et al*., 2000).

CONCLUSION

The findings of the present study clarifies that flavonoid compounds present in the lead extract may be responsible for the observed activity. Thus a few traditional Indian medicinal plants, particularly *Eugenia jambolana* seems as potential α-amylase inhibitor in the management of diabetes.

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