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In Vitro comparisons of anti-diabetic activity of flavonoids and crude extracts of *Azadiracta indica* A Juss

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Abstract

Indian medicinal plants used in the Ayurveda traditional system to treat diabetes are a valuable source of novel anti-diabetic agents. Alpha amylase inhibitors offer an effective strategy to lower the level of postprandial hyperglycemia via control of starch breakdown. Different crude extracts of *Azadiracta indica* A Juss have been considered as hypoglycemic agents. In this study, we compared the alpha amyse inhibitory activities of flavonoids with different crude extracts isolated from *Azadiracta indica* A Juss collected from Jaipur and Bharatpur districts of Rajasthan which sears approximately similar climatic conditions. Water, methanol, ethanol, acetone, toluene and petroleum ether were used for crude extraction. Alpha amylase inhibitory activities were evaluated by both qualitative and quantitative assays. Results showed that flavonoids have very high anti-diabetic potentials than crude extracts. IC_{50} values of flavonoids are 0.009g/ml and 0.006 g/ml in Jaipur and Bharatpur districts respectively while the IC_{50} values of other crude extracts are very high.

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INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia or increased blood glucose levels with disturbances in carbohydrate, fat and protein metabolism resulting

from absolute or relative lack of insulin secretion [1]. The frequency of this disorder is on the rise globally, is likely to hit 300 million by 2025 with India projected to have the largest number of diabetic cases [2].

It shows that an appropriate and effective step is needed to control the disease spectrum. One of the therapeutic approaches in type 2 diabetes is to lower the corresponding postprandial blood glucose values. Alpha amylase inhibitors play major role in the management of postprandial hyperglycemia [3]. α-amylase is a key enzyme in 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 postprandial hyperglycemia. It has been shown that activity of human α-amylase correlates to an increase in postprandial glucose level, the control of which is therefore an important aspect in treatment of diabetes [4]. Hence, retardation of starch digestion by inhibition of enzyme such as α-amylase would play a key role in the control of diabetes[5]. Inhibitors currently in clinical use for example, acarbose, miglitol, and voglibose are known to inhibit a wide range of glycosidases such as α-glycosidase and α-amylase [6]. But they have also exhibited a number of undesired side effects associated with their uses [7]. Therefore, the search for more safer, specific and effective hypoglycemic agents has continued to be an important area of investigation with natural extracts from readily available traditional medicinal plants offering great potential for discovery of the new anti-diabetic drugs.

Medicinal plants have been always an exemplary source of drugs. Traditional medicinal plants with their various biological constituents have been used effectively by the communities since long time to treat diseases. Plant extracts and bioactive herbal compounds have been reported scientifically for their biological activities [8]. Ethno botanical studies of traditional herbal remedies used for diabetes have identified more than 1,200 species of plants with hypoglycemic activity [9,10].

However, this traditional knowledge, derived empirically, has to be supported by scientific testing. WHO (World Health Organization) (1980) has recommended the evaluation and mechanistic properties of the plants effective in such systems [11,12]. The search for new pharmacologically active agents obtained by screening natural sources such as medicinal plants or their extracts can lead to potent and specific inhibitors for α-amylase [13].

Leaves of *Azadiracta indica* A juss have been reported to possess hypoglycemic activity. Alpha amylase inhibitory activity of *Azadiracta indica* A juss have been reported for various crude extracts. In this study, we compared the anti-diabetic potentials of flavonoids with the crude extracts of leaves of *Azadiracta indica* A juss .

MATERIALS AND METHODS

Plant material

Carefully inspected healthy plants were selected from different localities of Jaipur and Bharatpur districts in October 2011. All selected plants were botanically identified and authenticated. Leaves of these plants were dried at room temperature (27-30C) for 25-30 days maintaining hygienic conditions. After complete drying each plant material were grounded to form powder using a domestic electric grinder and then stored in brown bottles to conduct the experimental protocols.

Preparation of extracts

Leaves powder was taken in round bottom flask in different solvents. 30 g powder was taken in each flask and water, methanol, ethanol, acetone, toluene and petroleum ether were used as solvent. Dried material and solvents were taken in 1:10 ratio. Those were kept at soxhlet unit for 24 hours. Then each extracts were filtered. The filtrate was subjected to evaporation to obtain dried extract. The percentage yield of each dried plant extract was calculated.

Flavonoids extraction

Selected plant parts were separately washed with sterilized water; shade dried, and finely powdered using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan [14]. Hundred grams of each finely powdered sample was soxhlet extracted with 80% hot methanol (500ml) on a water bath for 24 h and filtered. Filtrate was re- extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, where as ethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. Ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for removal of bounded sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract thus obtained was washed with distilled water to neutrality. Ethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried and weighed.

In vitro α-amylase inhibitory assay

Starch iodine color assay

Screening of plant extracts for α-amylase inhibitors were carried out in test tubes according to Xiao *et al.* with slight modifications based on the starch -iodine test [15]. The total assay mixture was composed of 120 µl 0.02M sodium phosphate buffer (pH 6.9 containing 6 mM Sodium chloride), 1.5 ml of salivary amylase and plant extracts at a concentration from 0.3-1.5 $mgml^{-1}$ (w/v) were incubated at 37°C for 10 min. Then soluble starch (1%, w/v) was added to each reaction well and incubated at 37°C for 15 min. 1 M HCl (60 µl) was added to stop the enzymatic reaction, followed by the addition of 300 µl of iodine reagent (5 mM I_2 and 5 mM KI). The colour change was noted and the absorbance was read at 620 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 without the enzyme were also included. A dark-blue colour indicates the presence of starch; a yellow colour indicates the absence of starch while a brownish colour indicates partially degraded starch in the reaction mixture. In the presence of inhibitors from the extracts the starch added to the enzyme assay mixture is not degraded and gives a dark-blue colour complex whereas no colour complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase.

3, 5-dinitrosalicylic acid assay

The inhibition assay was performed using the chromogenic DNSA method [16]. The total assay mixture composed of 500 µl of 0.02 M Sodium phosphate buffer (pH 6.9 containing 6 mM Sodium chloride), 1ml of salivary amylase and 400 µl extracts at concentration from 0.3-1.5 $mgml^{-1}$(w/v) were incubated at 37°C for 10 min. After pre-incubation, 580 µl of 1% (w/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 were measured at 540 nm. The control represented 100% enzyme activity and did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls with the extract in the reaction mixture except for the enzyme were also included.

The % inhibition of alpha amylase was calculated as follows:

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

% Inhibition in the α-amylase activity= (100–% Relative enzyme activity).

Statistical Data Analysis

All experiments were performed in 3 different sets with each set in triplicates. The data are expressed as

mean ± SEM (standard error of the mean). Statistical difference, ANOVA and linear regression analysis were performed using Graph pad prism 5 statistical software. 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 extract, containing the α-amylase inhibitor that inhibited 50% of the alpha amylase activity.

Table 1: Alpha amylase Inhibitory activity of different extracts of *Azadiracta indica* A Juss collected from Jaipur district

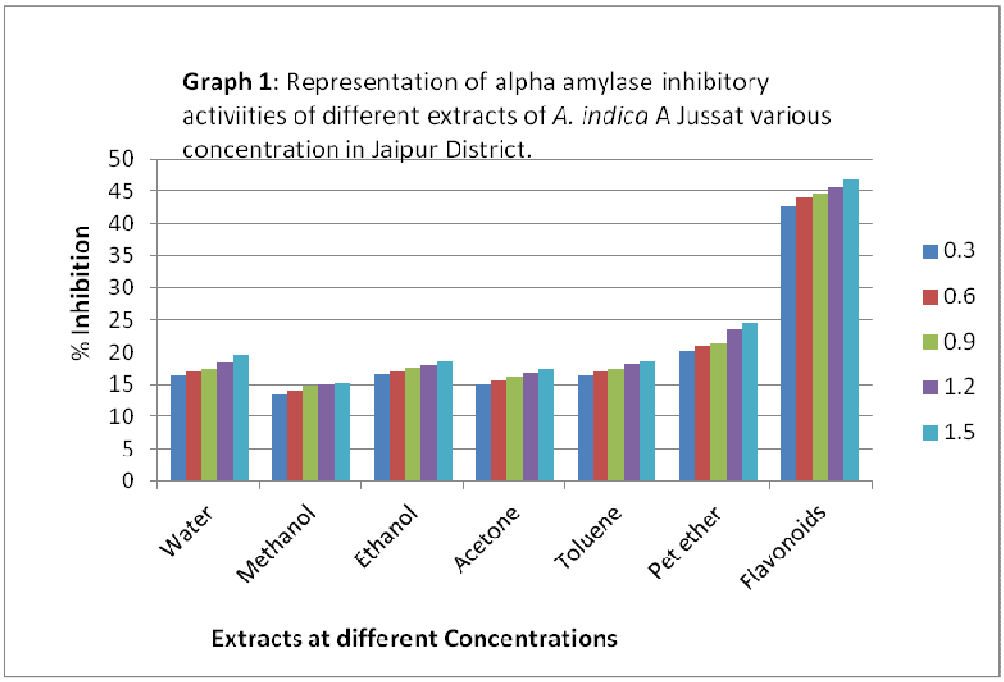
Extract	Concentration (mg/ml)	% Inhibition	Regression equation	IC_{50} value (g/ml)
Flavonoids	0.3	42.74±1.03	Y= 4.515+.0122x	0.0094
	0.6	43.95±0.13		
	0.9	44.57±0.11		
	1.2	45.63±0.13		
	1.5	46.85±0.13		
Water	0.3	16.43±0.12	Y= 3.489+0.206x	21.37
	0.6	17.05±0.23		
	0.9	17.53±0.11		
	1.2	18.33±0.21		
	1.5	19.47±0.20		
Methanol	0.3	13.35±0.13	Y= 3.411+0.184x	426.57
	0.6	14.14±0.16		
	0.9	14.75±0.12		
	1.2	15.01±0.12		
	1.5	15.16±0.17		
Ethanol	0.3	16.53±0.12	Y= 3.651+0.144x	2290.86
	0.6	17.03±0.20		
	0.9	17.63±0.11		
	1.2	18.06±0.26		
	1.5	18.53±0.14		
Acetone	0.3	15.12±0.11	Y= 3.566+0.158x	1174.89
	0.6	15.65±0.08		
	0.9	16.15±0.21		
	1.2	16.98±0.15		
	1.5	17.52±0.06		
Toluene	0.3	16.35±0.07	Y= 3.651+0.144x	2290.86
	0.6	17.04±0.19		
	0.9	17.54±0.12		
	1.2	18.12±0.16		
	1.5	18.53±0.10		
Pet ether	0.3	20.36±0.13	Y= 3.692+0.185x	11.74
	0.6	20.93±0.18		
	0.9	21.37±0.11		
	1.2	23.65±0.12		
	1.5	24.57±0.19		

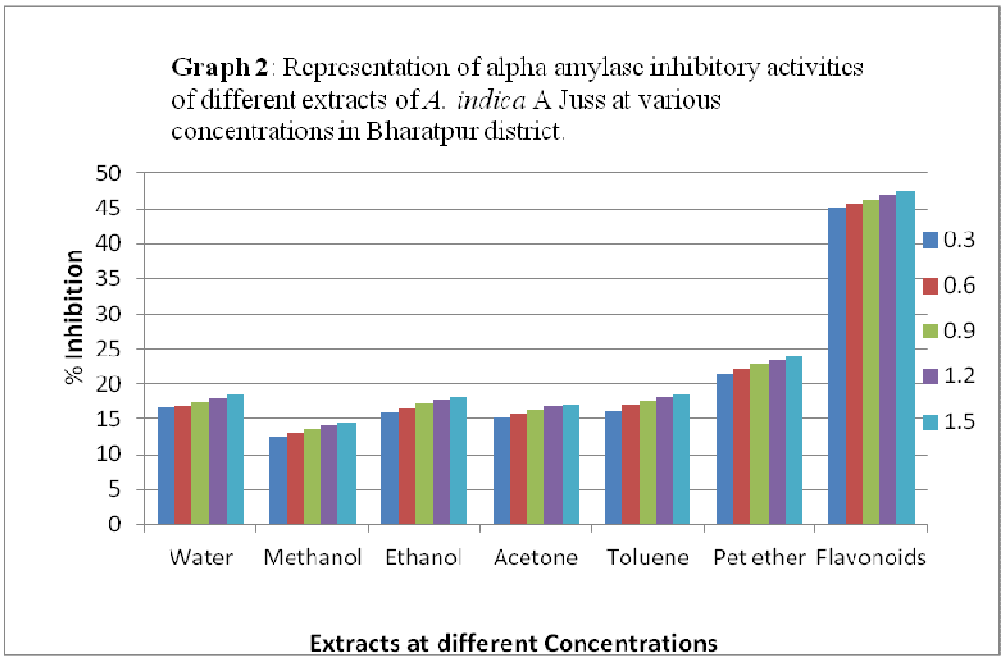
Notes: Values are given as mean±SD (n=3). Values in each line followed by different letters are significantly different ($\rho < 0.05$).

Table 2: Alpha amylase Inhibitory activity of different extracts of Azadiracta indica A Juss collected from Bharatpur district.

Extract	Concentration (mg/ml)	% Inhibition	Regression equation	IC_{50} value (g/ml)
Flavonoids	0.3	45.11±0.25	Y= 4.62+0.1x	.006309
	0.6	45.73±0.13		
	0.9	46.20±0.24		
	1.2	46.82±0.20		
	1.5	47.40±0.19		
Water	0.3	16.63±0.10	Y= 3.663+0.138x	4786.300
	0.6	16.95±0.11		
	0.9	17.32±0.21		
	1.2	17.84±0.14		
	1.5	18.52±0.16		
Methanol	0.3	12.53±0.13	Y= 3.266+0.202x	380.189
	0.6	13.10±0.16		
	0.9	13.54±0.20		
	1.2	14.09±0.32		
	1.5	14.44±0.14		
Ethanol	0.3	15.95±0.17	Y= 3.762+0.101x	1778279.410
	0.6	16.56±0.15		
	0.9	17.18±0.13		
	1.2	17.65±0.12		
	1.5	18.07±0.16		
Acetone	0.3	15.25±0.12	Y= 3.566+0.158x	1174.897
	0.6	15.87±0.15		
	0.9	16.33±0.21		
	1.2	16.84±0.27		
	1.5	17.12±0.23		
Toluene	0.3	16.22±0.16	Y= 3.651+0.144x	2290.867
	0.6	16.97±0.14		
	0.9	17.64±0.14		
	1.2	18.13±0.18		
	1.5	18.55±0.18		
Pet ether	0.3	21.57±0.10	Y= 3.856+0.135x	295.120
	0.6	22.24±0.17		
	0.9	22.94±0.18		
	1.2	23.33±0.26		
	1.5	23.94±0.17		

Notes: Values are given as mean±SD (n=3).Values in each line followed by different letters are significantly different ($\rho < 0.05$)





RESULTS AND DISCUSSION

Drugs that reduce postprandial hyperglycemia by suppressing hydrolysis of starch such as alpha amylase inhibitors have been found useful in the control of diabetes mellitus [17]. Many herbal extracts have been reported for their anti-diabetic activities and are currently being used in Ayurveda for the treatment of diabetes. However, such medicinal plants have not gained much importance as medicines due to the lack of sustained scientific evidences (18).

In the present study, different extracts of leaves of Azadiracta indica A juss collected from different localities of Jaipur and Bharatpur districts were evaluated for their respective α-amylase activity. The results showed that flavonoids showed the highest

percentage inhibition on α-amylase activity. This may be due to the presence of potential α-amylase inhibitors. Petroleum ether extracts showed moderate alpha amylase inhibitory activity while the other crude extracts showed very low inhibitory activity on alpha amylase.

The inhibitory activity and IC50 values of all extracts at different concentration of Azadiracta indica A Juss in Jaipur and bharatpur districts have been enlisted in Table1 and Table2 respectively.

Graph 1 and Graph 2 show comparative representation of % inhibition of alpha amylase by different extracts at different concentrations in Jaipur and Bharatpur districts respectively.

CONCLUSIONS

Azadiracta indica A juss plant used for the study is common food plant and is locally approved as plant having traditional values. The results of this study indicate that leaves of this plant possess potent inhibitory activity on salivary amylase. IC50 values of flavonoids are much lower than that of other crude extracts which have been reported earlier. Thus these extracts might help in identification of new lead molecules for natural amylase inhibitors. However, isolation and characterization of the active compound associated with amylase inhibition have to be carried out to confirm these observations.

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