ORIGINAL ARTICLE

Comparative study of alpha amylase inhibitory activity of flavonoids of *Vitex negundo* Linn. and *Andrographis paniculata* Nees

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Background: One important therapeutic approach for the treatment of Type 2 Diabetes Mellitus is by decreasing the postprandial increase of glucose. This is possible by inhibiting certain carbohydrate hydrolyzing enzymes like alpha-amylase. **Aim:** The objective of the present study was to evaluate the alpha amylase inhibitory activity of flavonoids extracts of different parts of *Vitex negundo* Linn and *Andrographis paniculata* Nees. **Materials and Methods:** In the present study, the percentage inhibitory effect of flavonoids isolated from different parts of *Vitex negundo* and *Andrographis paniculata* were studied with salivary alpha amylase and starch as substrate using chromogenic DNSA method and starch iodine method for qualitative estimation. **Statistical 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 and linear regression analysis were performed using Graph pad prism 5 statistical software. Statistical analysis was performed for ANOVA (analysis of variance) using Statistical Package for Social Sciences (SPSS) version 11.5. Values of *P* which were ≤0.05 were considered as significant. **Results and Conclusion:** The results clearly indicated that highest inhibition (62.22%) was obtained at the concentration of 1.5 mg/ml of flavonoids extracted from the leaves of *A. paniculata*, with an IC_{50} value of 0.004 mg/ml. Except *Vitex* leaf flavonoids extract, all other tested flavonoids of different parts of both the plants have shown more than 50% inhibition of alpha amylase activity. Thus the results are clearly indicating that the flavonoids of *V. negundo* and *A. paniculata* might be effective in lowering post prandial hyperglycemia.

Key words: Alpha amylase inhibition, *Andrographis paniculata,* flavonoids, post-prandial hyperglycemia, *Vitex negundo*

INTRODUCTION

Diabetes mellitus (DM) is a major health problem affecting major populations worldwide. It is a common irreversible metabolic disorder characterized by increase in blood glucose level and changes in carbohydrate, fat, and protein metabolism, as well as deficiencies in insulin secretion or insulin action or both.[1] Incidence of DM is increasing considerably from about 171 million people in 2,000 to about 366 million by 2030.[2]

One important therapeutic approach for treating diabetes is by decreasing the post-prandial increase in blood glucose level. Alpha amylase inhibitors play major role in the management of post-prandial hyperglycemia. It inhibits the action of alpha amylase enzyme leading to a reduction in starch hydrolysis to maltose and consequentially lower post-prandial hyperglycemia.[3] Various medications are available for the treatment of Type 2 diabetes such as biguanides, sulfonylureas, thiozolidinediones, etc. But they have also exhibited a number of undesired side effects associated with their uses and thus suggesting other effective alternatives. Medicinal plants have been an exemplary source in the treatment of various diseases including DM particularly in developing countries like India, because of high cost and poor availability of current therapies.[4] Alarcon-Aguilara *et al.*,[5] reported more than 800 plants are having anti-diabetic potentials. Plant extracts or bio-active herbal compounds have been reported scientifically for their biological activities. Administration of sulfur-containing amino acid isolated from *Alliumcepa* Linn called S-methyl cysteine sulfoxide (at a dose of 200 mg/kg for 45 days) showed anti-diabetic and hypolipidemic effects in alloxanized rats.[6] Hydro alcoholic extract of *Azadirachta indica* showed hypoglycemic and anti-hyperglycemic effects in normal, glucose-fed, and streptozotocin-induced diabetic rats.[7] Mangiferin from stem bark of *Mangifera indica* Linn showed anti-diabetic and anti-atherogenic effects.[8] Whole plants of *Phyllanthus amarus* showed anti-hepatitis and anti-diabetic activities due to the presence of chemical compounds such as phyllanthin, hypophyllanthin, phylletetralin, nirtetralin lintetralin, quercetin, and rutin.[9] *Tinospora cordifolia* leaves

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exhibited anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, and anti-malarial activity due to the presence of alkaloids, diterpenoids lactones, glycosides, steroids, and polysaccharides.[10] The ethno botanical information reports about 800 plants may possess anti-diabetic potentials.

This study is designed to study the alpha amylase inhibitory potential of two traditionally known medicinal plants of India namely *Vitex negundo* Linn and *Andrographis paniculata* Nees. These plants were chosen because their medicinal values have been emphasized by Ayurveda; however, there are lack of scientific investigations and evaluation of alpha amylase inhibitory activity of flavonoids of these plants.

MATERIALS AND METHODS

Plant Collection and Authentication

Different parts of *V. negundo* L. (Leaf, stem, root, and flower buds) and *A. paniculata* (whole aerial part, leaf, and root) were collected in the month of September-October and January-February 2010, respectively, from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at department of Botany, university of Rajasthan and (voucher specimen no: RUBL20838, RUBL20873) were submitted to the herbarium, Botany department, university of Rajasthan.

Preparation of Extracts

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, 1969.[11] Hundred grams of each finely powdered sample were soxhlet extracted with 80% hot methanol (500 ml) 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, whereas 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 in vacuo and weighed. The extracts were stored at 4°C.

Salivary α-amylase Inhibition Assays

Starch-iodine color assay

Screening of plant material for alpha amylase inhibitors was carried out according to Xiao *et al*., based on the starch iodine test.[12] The total assay mixture was composed of 120 µl 0.02 M sodium phosphate buffers (pH 6.9 containing 6 mM sodium chloride), 1.5 ml of salivary alpha amylase enzyme, and plant extracts at concentration of 0.5-1.5 mg/ml^1 (w/v) were incubated at 37°C for 10 min. Then soluble starch (1%, w/v) was added to each reaction set 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 color 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 color indicates the presence of starch; a yellow color indicates the absence of starch, while a brownish color 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 color complex, whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolyzed by α-amylase.

3,5-dinitrosalicylic Acid Assay

The inhibition assay was performed using the chromogenic DNSA method.[13] The total assay mixture composed of 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 1 ml of salivary alpha amylase enzyme, and extracts at concentration of 0.5-1.5 mg/ml^1 (w/v) were incubated at 37°C for 10 min. After pre-incubation, 580 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 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 with the extract in the reaction mixture except for the enzyme were also 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.

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 salivary alpha amylase activity.

The other quantifiers were 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 three different sets with each set in triplicates. The data are expressed as mean ± standard error of the mean (SEM). Statistical difference and linear regression analysis were performed using Graph pad prism five statistical software. Statistical analysis was performed for analysis of variance using SPSS version 11.5. $P \leq 0.05$ was considered significant.

RESULTS

The results showed that various extracts of selected plants exhibited different degree of alpha amylase inhibitory activities by assay using starch as a substrate [Table 1]. Flavonoid extracts of different parts of *V. negundo* have shown significant percentage inhibition of alpha amylase activity [Figures 1 and 2]. Highest inhibition was shown by flavonoids of stem and flower, i.e., 55.10% and 53.58%, respectively, at the concentration of 1.5 mg/ml, with an IC_{50} value of 0.08 mg/ml and 0.5 mg/ml ($P \leq 0.05$). Whereas for plant *A. paniculata*, flavonoid extracts of leaf have shown tremendous alpha amylase inhibitory effect of 62.22% at 1.5 mg/ml concentration, with a very low IC_{50} value of 0.004 mg/ml ($P \leq 0.05$) [Figure 2]. Except flavonoid extracts of leaves of *V. negundo*, all other parts of both the tested

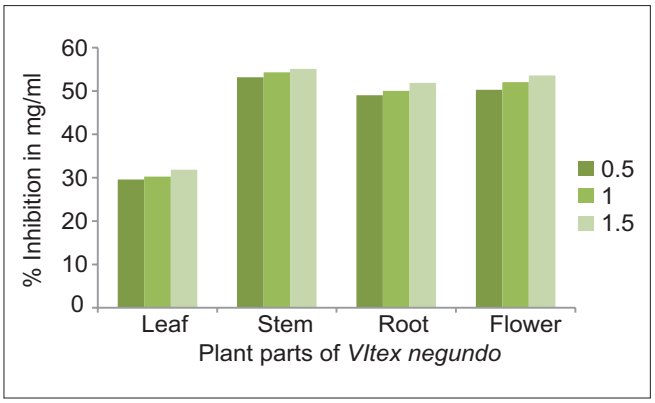

Figure 1: Representation of % inhibition of α-amylase activity of different concentration of various parts of *Vitex negundo* Linn

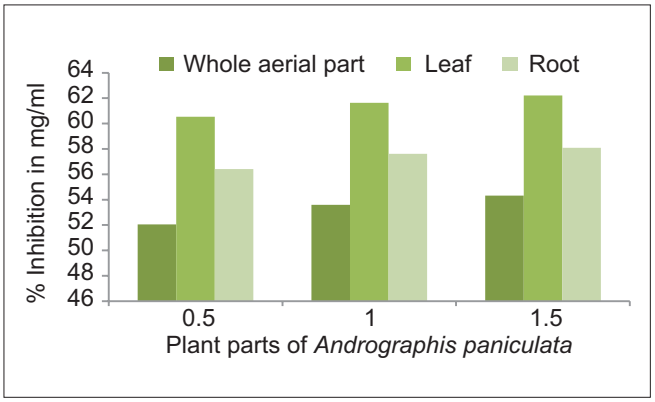

Figure 2: Representation of % inhibition α-amylase activity of different concentration of various parts of *Andrographis paniculata* Nees

Table 1: Percentage inhibitory effects on alpha amylase activity of flavonoids extracts of different parts of *Vitex negundo* and *Andrographis paniculata*

Plant	Plant part	Concentration in mg/ml	% inhibition	Log dose	Emprical probit	Regression equation	IC_{50} in mg/ml
Vitex negundo Linn.	Leaf	0.5	29.60±0.078	2.6990	4.45	*Y*=4.013+0.16x	1445.43
		1.0	30.26±0.180	3.000	4.48		
		1.5	31.83±0.023	3.1761	4.53		
	Stem	0.5	53.16±0.023	2.6990	5.08	*Y*=4.806+0.101x	0.08
		1.0	54.32±0.010	3.000	5.10		
		1.5	55.10±0.208	3.1761	5.13		
	Root	0.5	49.03±0.802	2.6990	4.97	*Y*=4.533+0.16x	0.8
		1.0	50.02±0.001	3.000	5.00		
		1.5	51.87±1.02	3.1761	5.05		
	Flower	0.5	50.30±1.04	2.6990	5.00	*Y*=4.548+0.167x	0.5
		1.0	52.05±0.09	3.000	5.05		
		1.5	53.58±0.003	3.1761	5.08		
Andrographis paniculata Nees	Whole aerial part	0.5	52.05±0.020	2.6990	5.05	*Y*=4.804+0.086x	0.2
		1.0	53.60±0.015	3.000	5.03		
		1.5	54.32±0.033	3.1761	5.10		
	Leaf	0.5	60.54±0.050	2.6990	5.25	*Y*=4.917+0.123x	0.004
		1.0	61.63±0.225	3.000	5.28		
		1.5	62.22±0.076	3.1761	5.31		
	Root	0.5	56.41±0.023	2.6990	5.15	*Y*=4.869+0.104x	0.017
		1.0	57.61±2.88	3.000	5.18		
		1.5	58.09±2.88	3.1761	5.20		

The data is indicated as the mean±SEM; (*n*=3), One way analysis of variance was used which show significant difference with respect to control ($P \leq 0.05$), IC – Inhibitory concentration

plants have shown excellent inhibition of alpha amylase activity, i.e., more than 50%.

DISCUSSION

Many herbal extracts have been reported for their anti-diabetic activities and being used in Ayurveda for the treatment of diabetes. In Type 2 DM, hyperglycemia is a condition characterized by an abnormal post-prandial increase in blood glucose level. Many plant extracts and natural products have been investigated with respect to suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine.[14] Alpha amylase catalyzes the hydrolysis of 1,4-glycosidic linkages of starch, glycogen, and various oligosaccharides into simpler sugars which can be readily available for the intestinal absorption. Thus, inhibition of alpha amylase enzyme in the digestive tract of human is being considered to be effective in controlling diabetes by decreasing the absorption of glucose from starch.[15] Ethanolic extract and andrographolide of *A. paniculata* have already been studied for alpha amylase and alpha glycosidase inhibitory activity, followed by a confirmatory *in vivo* study on rats.[16] Till now, studies for alpha amylase inhibitory activity were done using crude extracts with different solvents of different polarity, but this is the first time crude flavonoids have been tested for this particular action. In this study, the enzyme inhibitory activity of crude flavonoids isolated from different parts of both the selected plants was investigated. Further studies are required to find out the mode of action of these plant extracts as alpha amylase enzyme inhibitors and to qualify the action of different constituents in the extract. Thus, this study indicated that flavonoid extracts of *V. negundo* and *A. paniculata* could be useful in management of post-prandial hyperglycemia. The results of this study direct researches to evaluate the therapeutic potential of flavonoids in the management of post-prandial hyperglycemia and Type 2 diabetes either alone or in a combinatorial therapy.

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