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ANTI-DIABETIC EVALUATION OF VARIOUS EXTRACTS OF FLOWERS OF *ALLAMANDA CATHARTICA* LINN.

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ABSTRACT

Natural products and especially those derived from higher plants have historically played a pivotal role in the discovery of new pharmaceuticals. In this study, alkaloids, flavonoids, steroids, petroleum ether, methanolic and water extract from flowers of *Allamanda cathartica* Linn were extracted and tested for antidiabetic activity, with salivary alpha amylase and starch as substrate using chromogenic DNSA (2,4- Di nitro Salicylic Acid) method & Starch iodine method. All experiments were performed in 3 different sets each in triplicates. The data are expressed as mean ± SEM (standard error of the mean). The highest inhibition (49.32±0.93%) was obtained at concentration of 1.5 mg/ml of petroleum ether extract of flowers with an IC_{50} value of 1.764 g/ml. All the tested flower extracts have shown high inhibitory potential. Thus, flower extracts might be very useful in managing the postprandial hyperglycemia by inhibition of salivary alpha amylase enzyme.

INTRODUCTION

Plants have been used for health and medicinal purpose since immemorial time and possess rich and important sources of medicinal potentials since human civilization. Herbal medicines have often retained popularity for historical and cultural ingredients and are used primarily for treating mild and chronic ailments. India has an ancient heritage of traditional medicines; Materia Medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products[1]. The World health Organization (WHO) estimates that about 80% of the population living in the developing countries relies almost exclusively on traditional medicine for their primary healthcare needs[2]. Research on plants as potent medicinal agents to treat various human diseases was increased drastically in this decade[3]. More preference of plant-based medicines has outweighed synthetic medication in the treatment and management of different diseases[4]. The medicinal value of plants lies in their chemical substances which produces a definite physiologic action on the human body[5].The flower medicine was introduced by Dr. Edward Bach, called father of flower medicine. He was a bacteriologist and consultant of homeopathy in London, Not satisfied with homeopathic practice, he turned to flower medicines[6]. A number of researchers reported that various medical application of plant flowers[7]. There are about 400 families in the world of the flowering plants, of which at least 315 are represented by India[8]. Hence, this is a thrust area for evaluation and exploration of flower as medicinal agents. Diabetes mellitus has become a serious global health problem affecting about 10% population of the world. It is a carbohydrate metabolism disorder of endocrine system due to an absolute or relative deficiency of insulin secretion, action or both. The disorder affects more than 100 million people worldwide and by 2030 it is predicted to reach 366 million. The most prevalent form both in the global and Indian scenario is the non-insulin dependent diabetes mellitus (NIDDM 2) which is associated with elevated postprandial hyperglycemia[9]. Human α-amylase correlates to an increase in postprandial glucose level, the control of which is therefore an important aspect in treatment of diabetes[10]. Many diverse therapeutic strategies for the treatment of Type II diabetes are in use. The conventional available therapies for diabetes include stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues, oral hypoglycemic agents, such as biguanids and sulfonylureas and the inhibition of degradation of dietary starch by glycosidases such as α-amylase and α-glucosidase[11]. 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. Because of their non specificity in targeting different glycosidases, these hypoglycemic agents have their limitations and are known to produce serious

side effects. 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.

Anti-diabetic property of extracts of higher plants in India has been reported[12, 13]. Ethanol extract of stembark of *Mangifera indica* (Mango) reduced glucose absorption gradually during the whole perfusion period in Type II diabetic rats[14]. Aqueous extract of *S. cumini* or *Eugenia jambolana* seeds and *Psidium guajava* leaves showed higher inhibition against the porcine pancreatic amylase among the medicinal plants studied[15]. No literature related to antidiabetic activity of *Allamanda cathartica* Linn. was found. Hence, the present study has been carried out to evaluate antidiabetic potential of various extracts of flowers of an ornamental plant "*Allamanda cathartica* Linn." and this is done for the very first time.

MATERIAL AND METHODS

Plant collection: Flowers of *Allamanda cathartica* Linn. were collected from different localities of Jaipur. Shade dried, weighed and stored in containers for extraction purpose.

Extractions: Extraction of plant part in different polar, non polar solvents (Water, methanol and petroleum ether) and for their secondary metabolites (alkaloids, flavonoids and steroids) was carried out by well established methods.

Extraction of Secondary Metabolites

Chemicals

Acetic acid, Ethanol, NH_4OH, Methanol, Petroleum ether, Ethyl ether, Ethyl acetate, Sulphuric acid, HCl and Benzene.

Extraction of Alkaloids

Alkaloids were extracted from flowers of the selected plant by well established method[16]. Finely powered sample (100g) of flowers was extracted in 20ml methanol after shaking for 15 min. After filtration, the filtrate was kept for drying. Thereafter residual mass was treated with 1% H_2SO_4 (5ml. 2 times).Extraction was then done in 10ml chloroform ($CHCl_3$) by using separating funnel. Organic layer of chloroform was rejected and aqueous layer was basified with 30% NH_4OH (P^H=9-10). Again, extraction was done in 10ml chloroform & organic layer of chloroform (lower layer) was collected in a flask, extraction was repeated with fresh chloroform, was dried in vacuo.

Extraction of Flavonoids

Flowers of selected plant were subjected to the flavonoid extraction following the method of Subramanian and Nagarjan[17]. One hundred gram of 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), diethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fraction was discarded as being rich in fatty substances whereas diethyl ether and ethyl acetate fraction were analyzed for free and bound flavonoids respectively. The ethyl acetate fraction of sample was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for removal of bound sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract obtained was washed with distilled water to neutrality. Diethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vacuo and weighed.

Extraction of Steroids

Steroids were extracted from flowers of the selected plant by well established method[18] after preliminary detection of steroids. Finely powdered sample (100g) of flowers was extracted in petroleum ether for 24hrs.After filtration; residual mass was treated with 15% ethanolic HCl for 4hr. Extraction was then done in ethyl acetate followed by washing in dis. water to neutralize the extract. Neutral extract was then passed over Sodium sulphate to remove moisture contents and was dried in vacuo. Chloroform was used for reconstitution of extract, filtered, dried and stored for further use.

Extraction of crude extracts in polar and non polar solvents

Dry plant material (20 gm each) was taken separately in round bottomed flask in different polar & non polar solvents (water, methanol and petroleum ether) in the ratio of 1:10. Soxhlet extraction was carried out for 24 hours and filtered. Each filtrate was subjected to evaporation to obtain crude dried extract which was weighed and calculated for each gram plant material.

In vitro salivary α amylase inhibitory assay:

1. Starch – Iodine color assay:

Reagents: Starch solution (1%), Phosphate buffer of pH 6.9 and of 0.02 molarity, Iodine reagent, Salivary alpha amylase enzyme.

Procedure: Screening of plant extracts for α-amylase inhibitory activity was carried out in test tubes following the method of Xiao Z[19] with slight modifications based on the starch iodine test. Total assay mixture 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 of concentration range 0.5-1.5 $mgml^{-1}$ (w/v) were incubated at 37°C for 10 min. Soluble starch (1% w/v) was then added to each reaction mixture and were incubated at 37°C for 15 min. Thereafter 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). Colour change was observed and the absorbance was recorded at 620 nm. Reaction tubes of control representing 100% enzyme activity which did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls without the enzyme were also examined. Appearance of 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 was not degraded and gave dark-blue colour complex whereas no coloured complex was developed in the absence of the inhibitor, indicating that starch was completely hydrolysed by α-amylase.

2. Glucose-DNSA color assay:

Reagents: DNSA (2, 4- Di nitro salicylic acid) reagent, Phosphate buffer of pH 6.9 and of 0.02 molarity, 1% Starch solution, Salivary alpha amylase enzyme.

Procedure: Inhibition assay was performed using chromogenic DNSA method[20]. 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 of concentration ranging from 0.5-1.5 $mgml^{-1}$(w/v) was incubated at 37°C for 10 min. After pre-incubation, 580 µl of 1% (w/v) starch solution was added to each tube and were subjected to incubation at 37°C for 15 min. Reaction was then terminated by adding1.0 ml DNSA reagent and each tube was placed in boiling water bath for 5 min., cooled to room temperature and the absorbance was measured at 540 nm. Control containing no plant extracts showed 100% enzyme activity. 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 (negative control). Percent inhibition of alpha amylase was calculated as follows:

Percent Relative enzyme activity= (enzyme activity in test sample with extract/enzyme activity in control)*100.

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

Statistical Data Analysis

All experiments were performed in three different sets, each in triplicate. 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.

RESULTS

Results revealed that various extracts of flowers of *Allamanda cathartica* L. exhibit alpha amylase inhibitory activity of different level (Table 1). Petroleum ether extract of flowers were found to have maximum inhibitory potential. Steroid, water & free flavonoid extract of flowers were also recorded to have good alpha amylase inhibitory potential. Alkaloid, bound flavonoid & methanolic extract of flowers were showed moderate alpha amylase inhibitory activity.

Extracts with high inhibitory effect on alpha amylase activity:

Petroleum ether extracts of flowers (at a concentration of 0.5- 1.5 mg/ml) showed highest alpha amylase inhibitory activity from 34.21±0.60% to 49.32±0.93% with IC_{50} value of 1.764 g/ml. At the same concentration, free flavonoid (% inhibition= 9.58±0.36% to 19.85±0.27%, IC_{50} value= 9.74 g/ml), steroid (% inhibition= 17.11±0.21% to 34.70±0.42%, IC_{50} value= 3.37 g/ml) & water (% inhibition= 15.37±0.56% to 30.3±0.79%, IC_{50} value= 4.13 g/ml) extract of flowers also exhibit good level of alpha amylase inhibition. (Table 2)

Extracts with moderate inhibitory effect on alpha amylase activity:

Alkaloid (% inhibition= 9.19±0.58% to 16.56±0.57%, IC_{50} value= 39.91 g/ml), bound flavonoid (% inhibition= 6.97±0.25% to 13.6±0.40%, IC_{50} value= 26.08 g/ml) & methanolic (% inhibition= 16.63±0.37% to 26.67±0.61%, IC_{50} value= 12.86 g/ml) extract of flowers were recorded to have moderate alpha amylase inhibitory activity. (Table 3)

The alpha amylase inhibitory activity of different extracts of flowers of *Allamanda cathartica* Linn. has been shown in figure 1.

DISCUSSION

In India, indigenous herbal remedies such as Ayurveda and other Indian traditional medicine have since ancient times used plants in treatment of diabetes[21]. Ethno botanical studies of traditional herbal remedies used for diabetes have identified more than 1,200 species of plants with hypoglycemic activity[22, 23]. Even though, these traditional practices are empirical in nature, over 200 million people in India with limited access to primary healthcare centers, depend on traditional system of medicine to cater to their healthcare needs[24]. However, this traditional knowledge, derived empirically, has to be supported by scientific testing. Hence, present study has been carried out to calculate IC_{50} values for determination of alpha amylase inhibitory activities of extracts of flowers of *Allamanda cathartica* Linn. Petroleum ether extract found to exhibit highest alpha amylase inhibitory potential. Investigation of specific components of the

extracts responsible for the inhibitory potential and mode of action of these plant extracts as amylase inhibitors will be the thrust area for further researches. Results of this study indicate the high inhibitory potential of the pet ether, steroid and water extracts of flowers of *Allamanda cathartica* Linn. So these extracts might be helpful in the discovery of novel & nature based antidiabetic drugs to manage the postprandial hyperglycemia.

CONCLUSION

Allamanda cathartica Linn. plant is an ornamental plant reported to have various traditional values related to medicinal purposes. Present study indicates the high alpha amylase inhibitory potential of flower extracts of this plant. IC_{50} value of pet ether, steroid and water extracts of flowers are lowest than other extracts of flowers, indicating high inhibitory potential of these extracts. Thus, the identification of new potent molecules for natural amylase inhibitors will be supported by results of the present investigation. After this study, another subject of interest for the researchers will be discovery of the active compound responsible for the amylase inhibition.

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TABLE 1: LEVEL OF ALPHA AMYLASE INHIBITORY ACTIVITY OF DIFFERENT EXTRACTS OF FLOWERS OF *A. CATHARTICA* LINN.

Name of plant part	**Name of extract**	**Level of inhibitory activity**
Flower	Alkaloid	+
	Steroid	++
	Free flavonoid	++
	Bound flavonoid	+
	Pet ether	++
	Methanol	+
	Water	++

Note: Level: (++) indicate extracts with high inhibitory effect, (+) indicate extracts with moderate inhibitory effect.

TABLE 2: EXTRACTS WITH MAXIMUM INHIBITORY EFFECTS ON THE ALPHA AMYLASE ACTIVITY

Sr. No.	**Name of extracts**	**Concentration (mg/ml)**	**% inhibition**	**Regression equation**	**IC_{50} Value**
1.	Petroleum ether extract	0.5	34.21±0.60	Y=4.809+0.775X	1.764
		1.0	41.11±0.95		
		1.5	49.32±0.93		
2.	Steroid	0.5	17.11±0.21	Y=4.388+1.158X	3.377
		1.0	25.83±0.15		
		1.5	34.70±0.42		
3.	Water extract	0.5	15.37±0.56	Y=4.313+1.116X	4.126
		1.0	25.97±0.25		
		1.5	30.3±0.79		
4.	Free flavonoid	0.5	9.58±0.36	Y=3.97+1.0422X	9.738
		1.0	15.55±0.35		
		1.5	19.85±0.27		

NOTE: Values are given as mean±SD (n=3). One way analysis of variance was used which show significant difference with respect to control ($P \leq 0.05$).

TABLE 3: EXTRACTS WITH MODERATE INHIBITORY EFFECTS ON THE ALPHA AMYLASE ACTIVITY

Sr. No.	Name of extracts	Concentration (mg/ml)	% inhibition	Regression equation	IC_{50} Value
1.	Methanolic extract	0.5	16.63±0.37	Y=4.239+0.686X	12.863
		1.0	21.23±1.07		
		1.5	26.67±0.61		
2.	Bound flavonoid	0.5	6.97±0.25	Y=3.789+0.855X	26.084
		1.0	12.3±0.30		
		1.5	13.6±0.40		
3.	Alkaloid	0.5	9.19±0.58	Y=3.86+0.712X	39.914
		1.0	12.31±0.36		
		1.5	16.56±0.57		

NOTE: Values are given as mean±SD (n=3). One way analysis of variance was used which show significant difference with respect to control (P≤0.05).

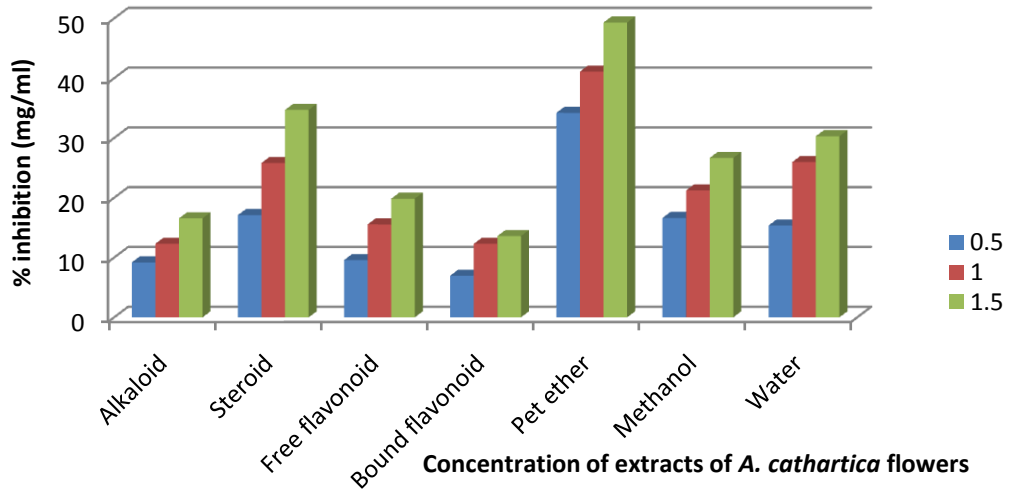

Figure 1: % Inhibition of alpha amylase by different extracts of *A. cathartica* Linn. flowers