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APPRAISAL OF HYPOGLYCEMIC NATURE OF EXTRACTS OF LEAVES AND FLOWERS OF AN ETHANOPHARMACOLOGICAL PLANT *NERIUM OLEANDER* LINN.

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Keywords:

Antidiabetic activity, Salivary alpha amylase, DNSA, Phosphate Buffer

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ABSTRACT

In present study, alkaloids, flavonoids, steroids, petroleum ether, methanolic and water extract from leaves & flowers of *Nerium oleander* 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 for leaf was found in its methanolic extract at the concentration of 1.5 mg/ml, with percent inhibition 57.14±1.03 % & an IC_{50} value of 1.296 g/ml while in case of flower, highest inhibition was obtained at 1.5mg/ml of bound flavonoid extract, with % inhibition 23.43±0.81% & IC_{50} value 3.489 g/ml and at 1.5 mg/ml of free flavonoid extract, with % inhibition 28.16±1.10% & an IC_{50} value 3.796 g/ml. 9 (6 of leaves & 3 of flowers) out of 14 tested extracts observed to exhibit good inhibitory potential but leaf extracts were found to be more potential than flowers extracts. Therefore, leaf extracts will be more fruitful in management of postprandial hyperglycemia and type2 diabetes with compare to flowers extracts.

INTRODUCTION

Diabetes mellitus is a collective form of various metabolic disorders affecting different organs of the body. The mode of action may by increasing the amount of insulin secreted by the pancreas (or) by increasing the sensitivity of target organs to insulin (or) decreasing the rate at which glucose is absorbed from the gastrointestinal tract[1]. It is caused primarily by a defect in the production of insulin by the islet cells of the pancreas resulting in an inability to use carbohydrates are characterized by hyperglycemia, glycosuria, polyuria, hyperlipemia (caused by imperfect catabolism of fats), acidosis, ketonuria, and a lowered resistance to infection. Glucose metabolism may get severely hindered due to improper insulin production from the pancreatic β-cells[2]. Several approaches were made to reduce the hyperglycemia, the hallmark of diabetes mellitus, with treatments such as sulfonylureas, which stimulates pancreatic islet cells to secrete insulin; meteoric, which acts to reduce hepatic glucose production; Glucosidase inhibitors, which interfere with glucose adsorption and insulin itself, which suppresses glucose production and augments glucose utilization[3]. The world health organization (WHO) estimates that about 80% of the population is still depends upon herbal medicines for their treatment of diseases due to easy availability, economic and less side effects when compared to allopathic system of medicines. Nearly 2000 of natural drugs are mentioned in Indian Materia Medica that have reported various pharmacological activities, out of these 1600 are from plant origin[4]. Herbal medicine have long history of use and better patient tolerance as well as acceptance. Medicinal plants have a renewable source, which are sustainable supplies of cheaper medicines. Availability of medicinal plants is not a problem especially in developing countries like India which is having rich agroclimatic, cultural and ethnic biodiversity. Herbal medicines may offer testimony of their safety and efficacy throughout the world. India is the largest producer of medicinal herbs and is called the botanical garden of the world[5,6,7,8,9]. Ethnobotanical information reports about 800 plants which possess anti-diabetic potential[10]. Plants contain glycosides, alkaloids, terpenoids, flavonoids, cartenoids, etc. that are frequently implicated as having antidiabetic activity[11]. Thus, study is focused on evaluation of antidiabetic activity of a plant "*Nerium oleander* Linn." belonging to family Apocynaceae. *Nerium oleander* extract was reported for improvement in activities of insulin, glucose and liver enzymes in hypoinsulinemic and hyperglycemic albino rats[12].*Nerium oleander* was identified to have potent wound healing

and anti diabetic activity[13]. *Nerium oleander* was reported for having anti-diabetic agents. These Pancreatic α-amylase inhibitors offer an effective strategy to lower the levels of post-prandial hyperglycemia via control of starch breakdown[14]. Ethanolic extract of flowers of *Nerium oleander* were reported to have anti- diabetic activity against alloxan induced diabetic rats[15]. *Nerium oleander* possesses potent anti-diabetic activity[16]. From the review of literature it is revealed that meager work has been carried out with this plant to evaluate the potency of this plant as a hypoglycemic agent. So in present study, extracts of leaves and flowers of this plant were selected for determination of their antidiabetic activity.

MATERIAL AND METHODS

Plant collection: Leaves & flowers of *Nerium oleander* Linn. were collected from different localities of Jaipur. Shade dried, weighed and stored in containers for extraction purpose.

Extractions: Extraction of plant parts 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 leaves & flowers of the selected plant by well established method[17]. Finely powered sample (100g) of leaves & flowers were extracted in 20ml methanol after shaking for 15 min. After filtration, the filtrates were kept for drying. Thereafter residual mass were 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 and was dried in vacuo.

Extraction of Flavonoids

Leaves & flowers of selected plant were subjected to the flavonoid extraction following the method of Subramanian and Nagarjan[18]. 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 leaves & flowers of the selected plant by well established method[19] after preliminary detection of steroids. Finely powdered sample (100g) of leaves & flowers were extracted in petroleum ether for 24hrs.After filtration; residual mass were 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[20] 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[21]. 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:

(i) % Relative enzyme activity= $\frac{\text{Enzyme activity in test sample with extract}}{\text{Enzyme activity in control}} * 100$

(ii) % 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

Different level of alpha amylase inhibitory activity was recorded for the various extracts of stem & root of *Nerium oleander* Linn. (Table 1). Free & bound flavonoids of both parts (Leaves & flowers) and crude extracts of leaves were found to have maximum inhibitory potential. Alkaloids of both parts were also recorded for good alpha amylase inhibitory activity while steroids of both parts and crude extracts of flowers were found to have insufficient inhibitory activity.

Extracts with high inhibitory effect on alpha amylase activity:

Leaf

Methanolic extract (at a concentration of 0.5- 1.5 mg/ml) showed the highest alpha amylase inhibition with high percent inhibition (19.76±0.67 % to 57.14±1.03%) and low IC_{50} value (1.296 g/ml). Bound flavonoid (% inhibition= 37.80±2.48 % to 49.26±1.09 %, IC_{50} value= 1.900 g/ml), free flavonoid (% inhibition= 28.07±2.11 % to 45.23±2.75 %, IC_{50} value= 2.021 g/ml), pet ether (% inhibition= 10.22±1.01 % to 29.89±2 %, IC_{50} value= 3.995 g/ml) & water (% inhibition= 14.29±1.12 % to 28.15±2.01 %, IC_{50} value= 7.443 g/ml) extracts were also exhibit good level of alpha amylase inhibition. (Table 2)

Flower

Bound flavonoid extract (at a concentration of 0.5- 1.5 mg/ml) showed the highest alpha amylase inhibition with high percent inhibition (4.45±0.70 % to 23.43±0.81 %) and low IC_{50} value (3.489

g/ml). Free flavonoid (% inhibition= 11.74±1.15 % to 28.16±1.10 %, IC_{50} value= 3.796 g/ml) extracts were also exhibit good level of alpha amylase inhibition. (Table 2)

Extracts with moderate inhibitory effect on alpha amylase activity:

Leaf

Alkaloid extract (% inhibition= 12±2 % to 28.17±0.66 %, IC_{50} value= 10.840 g/ml) was recorded to have moderate alpha amylase inhibitory activity.

Flower

Alkaloid extract (% inhibition= 3.2±0.91 % to 33.97±1.35 %, IC_{50} value= 17.894 g/ml) was recorded to have moderate alpha amylase inhibitory activity. (Table 3)

Extracts with insufficient inhibitory effect on alpha amylase activity:

Leaf

Steroids were recorded for insufficient inhibitory activity with low percent inhibition and high IC_{50} value.

Flower

Steroid & crude extracts (Petroleum ether, Methanol and Water) were found to have insufficient inhibitory activity with low % inhibition and high IC_{50} values.

The alpha amylase inhibitory activity of different extracts of leaves & flowers of *Nerium oleander* Linn. has been shown in figure 1 & figure 2, respectively.

DISCUSSION

World health organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes as they are effective, non-toxic, which less or no side effects and are considered to be a valuable source for the investigation of hypoglycemic agents[22, 23]. Several reviews on the plants used in the management of diabetes have been reported in the past[24, 25, 26, 27, 28, 29, 30, 31]. Plant products are known to be rich in phenolic compounds, flavonoids, terpenoids, coumarins and other constituents which reduce blood glucose levels[32, 33]. The variety of phytoconstituent classes and the wide differences in the molecular structure of the isolated compounds suggest the possibility of different mechanisms of action in lowering blood glucose[34]. The mode of action may by increasing the amount of insulin secreted by the pancreas (or) by increasing the sensitivity of target organs to insulin (or) decreasing the rate at which glucose is absorbed from the gastrointestinal tract. The current study strengthens the drug

development by using a medicinal plant. Present study is undertaken with leaves and flowers of a medicinal plant "*Nerium oleander* Linn." Deceleration of starch digestion by inhibition of α-amylase enzyme would play a key role in the control of diabetes[35]. Hence, calculations of IC_{50} values were done for determination of alpha amylase inhibitory activities of extracts. Bound flavonoid & methanolic extract of leaves and Free & bound flavonoids of flowers were found to exhibit most hypoglycemic potential. Results indicate that leaves are more hypoglycemic in nature than flowers but extracts of both parts are to be considered as therapeutically potential in managing the hyperglycemia.

CONCLUSION

It is concluded that extracts of leaves & flowers of *Nerium oleander* Linn. plant contains potent inhibitory activity on salivary alpha amylase enzyme, however, leaf extracts were observed to be more hypoglycemic than flower extracts. Various extracts with low IC_{50} values indicated their strong inhibition power. Although extracts of leaves were found to be more active than flower extracts, however extracts of both parts might play an important role in search of natural inhibitors. Hence more advanced studies are needed in order to identify the bioactive principles to treat diabetes. It is the responsibility of the scientific community to find appropriate mode of action & efficient bioactive compounds from plant sources and they should be necessarily formulated to cure diabetes.

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1: LEVEL OF ALPHA AMYLASE INHIBITORY ACTIVITY OF DIFFERENT EXTRACTS OF *N. OLEANDER* LINN.

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

Note: Level: Indication of inhibitory potential of extract
(++) indicated extracts with moderate inhibitory effects on alpha amylase.
(+) indicated extracts with minimum inhibitory effects on alpha amylase activity.
(-) indicated extracts with insignificant (no) inhibitory effects on alpha amylase activity.

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
Leaf					
1.	Methanol	0.5	19.76±0.67	Y=4.764+2.097X	1.296
		1.0	37.76±2.64		
		1.5	57.14±1.03		
2.	Bound flavonoid	0.5	37.80±2.48	Y=4.843+0.563X	1.900
		1.0	42.12±2.01		
		1.5	49.26±1.09		
3.	Free Flavonoid	0.5	28.07±2.11	Y=4.71+0.949X	2.021
		1.0	38.87±1.72		
		1.5	45.23±2.75		
4.	Pet ether	0.5	10.22±1.01	Y=4.102+1.493X	3.995
		1.0	14.22±0.83		
		1.5	29.89±2		
5.	Water	0.5	14.29±1.12	Y=4.157+0.967X	7.443
		1.0	16.48±1.50		
		1.5	28.15±2.01		
Flower					
1.	Bound flavonoid	0.5	4.45±0.70	Y=3.864+2.093X	3.489
		1.0	12.11±0.58		
		1.5	23.43±0.81		
2.	Free flavonoid	0.5	11.74±1.15	Y=4.244+1.305X	3.796
		1.0	25.46±0.69		
		1.5	28.16±1.10		

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
Leaf					
1.	Alkaloid	0.5	3.2±0.91	Y=3.523+1.427X	10.840
		1.0	6.37±0.72		
		1.5	12±2		
Flower					
1.	Alkaloid	0.5	28.17±0.66	Y=4.539+0.368X	17.894
		1.0	33.05±1.02		
		1.5	33.97±1.35		

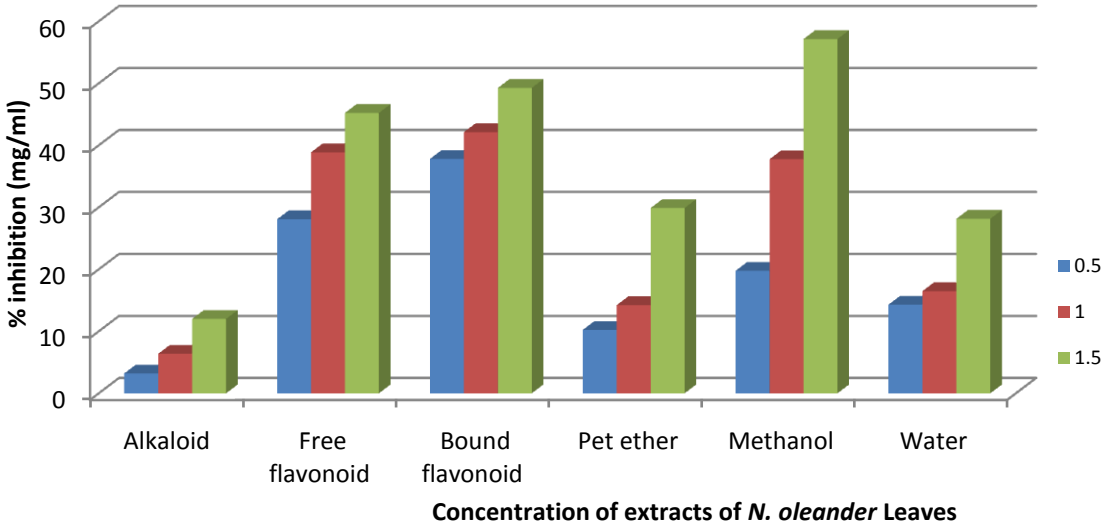

Figure 1: % Inhibition of alpha amylase by different extracts of *N. oleander* Linn. leaves

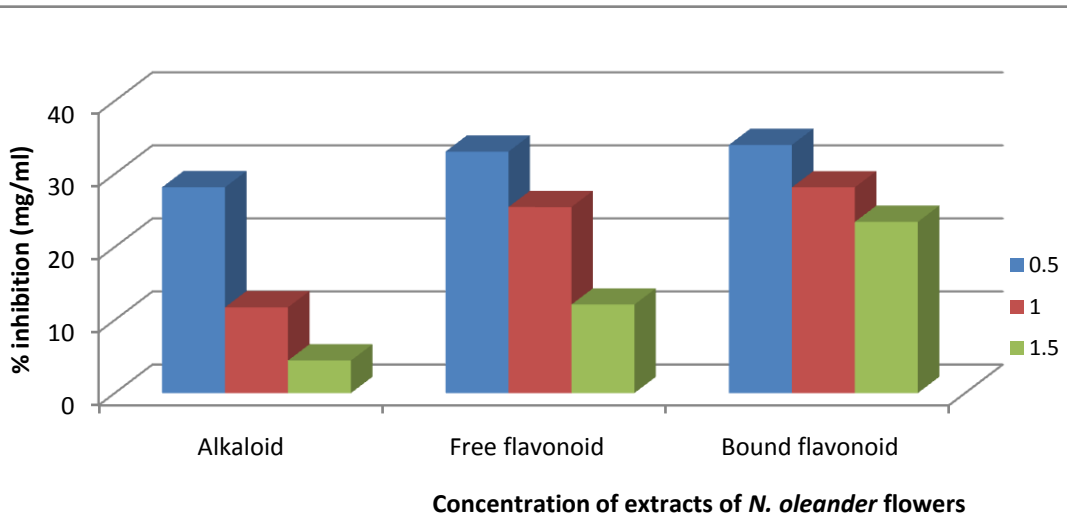

Figure 2: % Inhibition of alpha amylase by different extracts of *N. oleander* Linn. flowers