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CARISSA CARANDAS LINN.: EXTRACTION OF VARIOUS EXTRACTS OF LEAVES & ASSESSMENT OF THEIR HYPOGLYCEMIC POTENTIAL

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ABSTRACT

Diabetes mellitus (DM) is a major endocrine disorder, affecting approximately 5% of the world's population. Diabetes is characterized by abnormalities in carbohydrate, lipid and lipoprotein metabolisms, which leads to hyperglycemia. In this study, alkaloids, flavonoids, steroids, petroleum ether, methanolic and water extract from leaves of *Carissa carandas* 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 (44.2±1.10%) was obtained at concentration of 1.5 mg/ml of steroid of leaves with an IC_{50} value of 2.271 g/ml and 1.5mg/ml of alkaloids of leaves with % inhibition (43.92±2.68%) & IC_{50} value (2.798). All the tested leaf extracts have shown good inhibitory potential. Thus, leaf extracts might be helpful in management of postprandial hyperglycemia.

INTRODUCTION

Diabetes mellitus is a metabolic disorder, characterized by chronic hyperglycemia, with disturbances of carbohydrate, fat and protein metabolism, resulting defects in insulin secretion, insulin action, or both[1]. Diabetes related deaths are more common in the low and middle-income countries where more than 80% deaths occur[2].The World Health Organization projects that diabetes will be the 7th leading cause of death in 2030[3]. 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[4]. The growing public interest and awareness of natural medicines have led the pharmaceutical industry and academic researchers to pay more attention to medicinal plants[5]. The apparent reversal of trend from western to herbal medicine is partly due to the fact that synthetic drugs have always shown adverse reactions and other undesirable side effects. This has led to the belief that natural products are safer because they are more harmonious with biological systems. In addition, the cost of administering modern antidiabetic drugs is beyond the reach of people in the low income group and those living in the rural areas[6]. Indian traditional health care system uses a number of medicinal plants traditionally over 1000 years in herbal preparations. Medicinal plants, minerals and organic matter cover a major part of traditional medicines. Most of the Indian traditional medical practitioners formulate and dispense their own recipes. 21,000 plants are listed by the WHO, which are used for medicinal purposes around the world. Among these, 2500 species are in India, out of which, 150 species are used commercially on a fairly large scale. India is the largest producer of medicinal herbs and is called the botanical garden of the world[7, 8, 9, 10, 11]. Ethnobotanical information reports about 800 plants which possess anti-diabetic potential[12]. Plants contain glycosides, alkaloids, terpenoids, flavonoids, cartenoids, etc. that are frequently implicated as having antidiabetic activity[13]. Even though plant sources are potential antidiabetic drugs, they have not gained sufficient momentum among the scientific community. *Carissa carandas* was reported as anti-diabetic plant used in the treatment of diabetes mellitus[14].*Carissa carandas* Linn reported to have hypoglycemic potentials in normal and glucose loaded mice and rats. In another study, reported the antihyperglycemic effects of *Carissa carandas* Linn in the glucose induced hyperglycemia[15].Methanolic and ethyl acetate extract of unripe fruit of *Carissa carandas* were found to lower the elevated blood glucose levels[16]. Aqueous extract of *C. carandas* reported to have antidiabetic effect on alloxan induced and normoglycemic Wister

rats[17]. Literature indicates that only fruit part has been reported to have hypoglycemic potential. So this study has been carried out to explore the antidiabetic potential of leaf extracts of *Carissa carandas* Linn.

MATERIAL AND METHODS

Plant collection: Leaves of *Carissa carandas* 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 leaves of the selected plant by well established method[18]. Finely powered sample (100g) of leaves 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 and was dried in vacuo.

Extraction of Flavonoids

Leaves of selected plant were subjected to the flavonoid extraction following the method of Subramanian and Nagarjan[19]. 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 of the selected plant by well established method[20] after preliminary detection of steroids. Finely powdered sample (100g) of leaves 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[21] 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[22]. 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 leaves of *Carissa carandas* L. exhibit alpha amylase inhibitory activity of different level (Table 1). Steroids and alkaloids of leaves were found to have maximum inhibitory potential. Free flavonoid, bound flavonoid, methanolic & water extract of leaves were also showed good alpha amylase inhibitory activity while pet ether extract of leaves was found to have moderate alpha amylase inhibitory activity.

Extracts with high inhibitory effect on alpha amylase activity:

Steroid and alkaloid (at a concentration of 0.5- 1.5 mg/ml) showed the highest alpha amylase inhibition with high percent inhibition (24.68±3.45% to 44.2±1.10%) & (32.10±1.42% to 43.92±2.68%) and low IC_{50} value (2.27 g/ml) & (2.79 g/ml), respectively. Free flavonoid (% inhibition= 15.62±1.80% to 30.65±1.78%, IC_{50} value= 5.49 g/ml), bound flavonoid (% inhibition= 25.55±1.86% to 35.68±1.64%, IC_{50} value= 6.84 g/ml), methanolic (% inhibition= 21.72±1.75% to 34.75±1.63%, IC_{50} value= 5.63 g/ml) & water (% inhibition= 13.52±1.37% to 24.63±2.28%, IC_{50} value= 7.92 g/ml) extracts were also exhibit good level of alpha amylase inhibition. (Table 2)

Extracts with moderate inhibitory effect on alpha amylase activity:

Petroleum ether extract (% inhibition= 134.57±1.06% to 12.49±1.61%, IC_{50} value= 16.69 g/ml) of leaves were recorded to have moderate alpha amylase inhibitory activity. (Table 3)

The alpha amylase inhibitory activity of different extracts of leaves of *Carissa carandas* Linn. has been shown in figure 1.

DISCUSSION

Medicinal plants have been known for several years but they remains enigma for researchers. The feasibility of making efficient drugs from plant sources is very much near. As this chronic

hyperglycemic disease is accelerating, there is an emergency for finding herbal inhibitors. It is the responsibility of the scientific community to find efficient bioactive compounds from plant sources and they should be necessarily formulated to cure diabetes. 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[23]. Hence, retardation of starch digestion by inhibition of enzyme such as α-amylase would play a key role in the control of diabetes[24]. Therefore, we calculated IC_{50} values for determination of alpha amylase inhibitory activities of extracts of leaves of *Carissa carandas* Linn. Steroid and alkaloid extract were found to have highest hypoglycemic potential. Further researches are required to find out the mode of action of these plant extracts as amylase inhibitors and to identify the action of different constituents in the extract. Steroid and alkaloid extracts showed their therapeutic potential in the management of postprandial hyperglycemia and type 2 diabetes.

CONCLUSION

Carissa carandas is an important drug mentioned in the traditional medicinal texts. Results indicate that leaves of this plant exhibits potent inhibitory activity on salivary alpha amylase enzyme. IC_{50} value of steroid and alkaloid of leaves are lowest than other extracts of leaves, indicating high inhibitory potential of these extracts. Thus, these extracts might play an important role in identification of new lead molecule as natural amylase inhibitors. Discovery of the active compound responsible for the amylase inhibition will be another thrust area for further researches.

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TABLE 1: LEVEL OF ALPHA AMYLASE INHIBITORY ACTIVITY OF DIFFERENT EXTRACTS OF *C. CARANDAS* LINN.

Name of plant part	**Name of extract**	**Level of inhibitory activity**
Leaf	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.

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.	Steroid	0.5	24.68±3.45	Y=4.71+0.649X	2.271
		1.0	33.16±2.83		
		1.5	44.2±1.10		
2.	Alkaloid	0.5	32.10±1.42	Y=4.624+1.055X	2.798
		1.0	37±1		
		1.5	43.92±2.68		
3.	Free flavonoid	0.5	15.62±1.80	Y=4.274+0.981X	5.496
		1.0	21.07±1.01		
		1.5	30.65±1.78		
4.	Methanol	0.5	21.72±1.75	Y=4.489+0.612X	5.628
		1.0	26.41±2.22		
		1.5	34.75±1.63		
5.	Bound flavonoid	0.5	25.55±1.86	Y=4.432+0.757X	6.839
		1.0	27.95±2.65		
		1.5	35.68±1.64		
6.	Water	0.5	13.52±1.37	Y=4.146+0.95X	7.924
		1.0	19.36±1.13		
		1.5	24.63±2.28		

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.	Pet ether	0.5	4.57±1.06	Y=3.578+1.163X	16.699
		1.0	7.20±1.25		
		1.5	12.49±1.61		

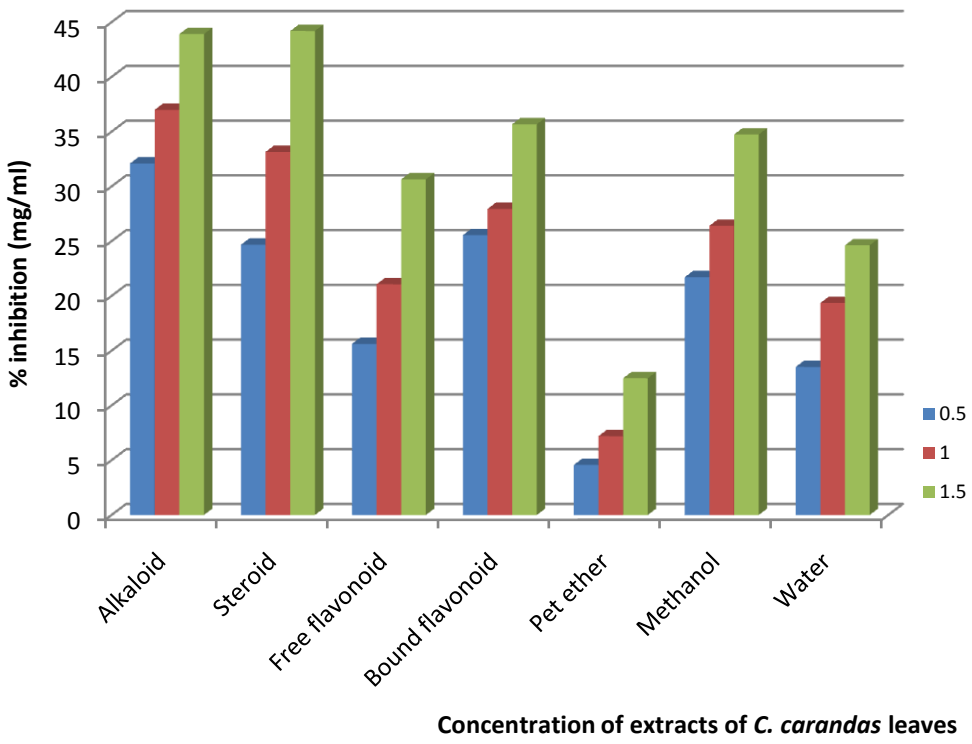

Figure 1: % Inhibition of aplha amylase by different extracts of *C.carandas* Linn. leaves