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EVALUATION OF ALPHA - AMYLASE AND ALPHA - GLUCOSIDASE INHIBITORY ACTIVITIES OF *RHIZOPHORA MUCRONATA*

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ABSTRACT: *Rhizophora mucronata* Lam. belongs to mangrove plants and antidiabetic activity is one of the traditional uses of *R. mucronata*. One of the anti-diabetic therapeutic strategies is inhibition of carbohydrate digesting enzymes such as alpha-amylase and alpha-glucosidase. Hence in the present study, 2 extract namely, aqueous and ethanol of *Rhizophora mucronata* Lam. were evaluated for their effect on alpha-amylase and alpha-glucosidase enzymes using *in vitro* assays. Ethanol extract showed the highest alpha-glucosidase inhibitory activity (IC_{50}= 9.45 µg/ml) than aqueous extract. Ethanol extract inhibited the enzyme more potently than the standard acarbose. Further, the antioxidant activity of extracts was monitored using DPPH assay, whereas, phenolic content was estimated using Folin-Ciocalteu reagent. The alpha-glucosidase inhibitory activity of *R. mucronata* correlated to antioxidant activity and phenolic content of extracts. However, these extracts showed no alpha-amylase inhibitory effect. Besides, *R. mucronata* has shown anti-HIV potential in our previous study. Oxidative stress and anti-HIV drugs are associated with diabetes. Our current and previous studies thus report anti-diabetic, antioxidant and anti-HIV activities of *R. mucronata*.

INTRODUCTION: Diabetes mellitus is a serious metabolic disorder worldwide and India is considered as the "Diabetes Capital" of the world [1]. The most prevalent form of diabetes affecting 90-95% of diabetics is type-2 which is associated with elevated postprandial hyperglycemia (PPHG) [2]. Type-2 diabetes has increased morbidity and mortality because of its chronic complications [3]. Postprandial glucose control has been proven to be important for prevention of diabetic complications [4]. One of the anti-diabetic therapeutic strategies is inhibition of carbohydrate digesting enzymes such as alpha-amylase and alpha-glucosidase [5].

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Alpha-amylase hydrolyzes complex starches to oligosaccharides, while, alpha-glucosidase hydrolyzes oligosaccharides to glucose and other monosaccharides. Inhibition of these enzymes produces postprandial anti-hyperglycemic effect by reducing the rate and extent of glucose absorption [6]. Currently, there are 5 classes of conventional anti-diabetic drugs; however, these drugs are associated with various side-effects [7]. Hence there is urgent need to identify and explore natural sources with fewer side-effects for such inhibitors.

In the present study, two extracts namely, aqueous and ethanol were prepared from *Rhizophora mucronata* Lam. They were evaluated for their effect on alpha-amylase and alpha-glucosidase enzymes. *R. mucronata* belongs to mangrove plants. The term mangrove is used to describe halophytic and salt-resistant marine tidal plants [8] which are also used in folklore medicine for

treatment of several diseases [9]. Some of the biological properties of *R. mucronata* have been reported previously [10] and antidiabetic activity is one of the traditional uses of *R. mucronata* which has been evaluated in the present study using different *in vitro* assays.

MATERIALS AND METHODS:

Collection of the material: *Rhizophora mucronata* Lam. was collected from Vikroli highway, Mumbai. The material was identified and authenticated by Dr. J. M. Pathak, Research Director (Pharmacognosy), Zandu Pharmaceuticals, Mumbai. The leaves were washed, shade-dried and powdered in a grinder mixer.

Preparation of extracts: The material was extracted in a Soxhlet apparatus with ethanol to obtain ethanol extract. Aqueous extract was obtained by plain decoction method using material with distilled water [11]. All the extracts were made free from solvents and percentage yield of individual extract was calculated. Extracts were kept at 4^{o}C until further use. Ethanol extract was reconstituted in 20% ethanol (as 20% ethanol was not inactivating the enzymes), whereas, aqueous extract was reconstituted in distilled water.

Alpha-Amylase Assay: Alpha-amylase assay of extracts was carried out according to method of Sudha *et al* [12] with slight modification. In a 96-well plate, reaction mixture containing 50µl phosphate buffer (50mM, pH= 6.8), 10µl alpha-amylase (10U/ml) [SRL] and 20µl of varying concentrations of extracts was pre-incubated at 37^{o}C for 10 min. Then 20µl soluble starch (0.05%) [HiMedia] was added as a substrate and incubated further at 37^{o}C for 15 min. The reaction was stopped by adding 20µl 1N HCl, followed by addition of 100µl iodine reagent (5mM I_2 and 5mM KI, stored in amber colored bottle). The absorbance was read at 620nm using Multimode Reader (Synergy HT, BioTek). Each experiment was performed in triplicates, along with appropriate blanks. Acarbose at various concentrations (10-100 µg/ml) was included as a standard. Acarbose was provided by Mr. Dnyaneshwar Nagmoti, Institute of Chemical Technology, Mumbai. Negative control without extracts was set up in parallel.

Alpha-Glucosidase Assay: Alpha-glucosidase assay of extracts was carried out according to method of Bachhawat *et al* [13] with slight modification. In a 96-well plate, reaction mixture containing 50µl phosphate buffer (50mM, pH= 6.8), 10µl alpha-glucosidase (1U/ml) [SRL] and 20µl of varying concentrations of extracts was pre-incubated at 37^{o}C for 15 min. Then 20µl p-nitrophenyl-α-D-Glucopyranoside (PNPG) (1mM) [SRL] was added as a substrate and incubated further at 37^{o}C for 30 min. The reaction was stopped by adding 50µl sodium carbonate (0.1M). The yellow color produced was read at 405nm using Multimode Reader (Synergy HT, BioTek). Each experiment was performed in triplicates, along with appropriate blanks. Acarbose at various concentrations (200-1000 µg/ml) was included as a standard. Negative control without extracts was set up in parallel. The result is expressed as percentage inhibition, which was calculated as,

Inhibition (%) = $A_{\text{Negative control}} - A_{\text{Test}}/A_{\text{Negative control}}$ X 100, where, A is absorbance. The result is also expressed as IC_{50} value.

DPPH radical-scavenging assay: The free radical scavenging activity of *R. mucronata* was measured by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) assay [14]. For this, 1 ml of DPPH solution (0.1mM) in methanol was added to different concentrations of extracts. After incubating for 30 minutes in dark, the absorbance was measured at 517nm using Multimode Reader (Synergy HT, BioTek). Ascorbic acid at various concentrations (3-7 µg/ml) was included as a standard. A negative control without extracts was run in parallel. The percent DPPH-scavenging activity was calculated as, DPPH scavenged (%) = $A_{\text{Negative control}} - A_{\text{Test}}/A_{\text{Negative control}}$ X 100, where, A is absorbance. The antioxidant activity of *R. mucronata* is also expressed as IC_{50} value.

Phenolic content estimation: The total phenolic content of *R. mucronata* was determined using Folin-Ciocalteu reagent according to the method of Pandima Devi *et al* [15]. Gallic acid at various concentrations (4-20 µg/ml) was included as a standard. All the determinations were done in triplicate. Mean values of triplicate determinations were used to plot the graph.

Total phenolic content was calculated from the equation (y= 0.045x, R^2= 0.997) obtained from the Gallic acid standard curve. The total phenolic content was expressed as Gallic acid equivalent (GAE) in mg/g of dry sample.

Statistical analysis: All the determinations were done in triplicate. Means, standard deviations and IC_{50} values were calculated using a Microsoft Excel program.

RESULTS AND DISCUSSION: Plants hold definite promises in the management of Diabetes mellitus [16-18]. In the present study, aqueous and ethanol extracts of *R. mucronata* were evaluated for their effect on alpha-amylase and alpha-glucosidase enzymes using *in vitro* assays. Porcine pancreatic alpha-Amylase (PPA) is closely related to human alpha-Amylase [12]. Hence PPA was used to evaluate inhibitory activity of *R. mucronata* extracts with starch as a substrate. The assay was based on starch-iodine color complex formation, whereas, alpha-glucosidase inhibitory activity was evaluated using p-nitrophenyl-α-D-Glucopyranoside (PNPG) as a substrate which was based on development of yellow color of p-nitro phenol. Ethanol extract showed the highest α-glucosidase inhibitory activity with IC_{50} value of 9.45 µg/ml (**Table 1**). Also, it has inhibited alpha-glucosidase more potently than the standard acarbose. However extracts of *R. mucronata* did not show any effect on alpha-amylase enzyme. Similar result has been reported by Kwon *et al* (inhibition of alpha-glucosidase enzyme, but no inhibitory activity on alpha-amylase enzyme by clonal extracts of herbs of Lamiaceae species) [19]. According to Mogale *et al* [20], natural inhibitors from plants are reported to have lower inhibitory effect against alpha-amylase and stronger inhibitory activity against alpha-glucosidase and our study supports this finding.

TABLE 1: EFFECT OF *R. MUCRONATA* ON ALPHA-GLUCOSIDASE

Extract	Concentration (µg/ml)	% Inhibition [Mean±SD]	IC_{50} (µg/ml)
Aqueous	200	21.26 ± 3.8	489.30
	400	37.29 ± 10.6	
	600	67.87 ± 1.2	
	800	84.12 ± 2.7	
	1000	90.80 ± 1.2	
Ethanol	6	19.73 ± 2.3	9.45
	8	37.55 ± 3.4	
	10	55.75 ± 6.0	
	12	77.90 ± 2.6	
	16	96.41 ± 3.2	
Acarbose (Standard)	200	34.21 ± 3.1	468.92
	400	47.07 ± 1.9	
	600	57.98 ± 7.2	
	800	63.14 ± 0.7	
	1000	78.76 ± 5.5	

The role of oxidative stress in diabetes and diabetic complications has been reported [21]. Antioxidants can scavenge free radicals and play important role in prevention of diabetes. Hence in present study, antioxidant effects of *R. mucronata* extracts were evaluated by testing their ability to bleach (purple to yellow color) the stable DPPH radical which is a widely used rapid and simple method. Ethanol extract showed potent inhibitory activity than aqueous extract with IC_{50} value of 34.82 µg/ml (**Table 2**). The alpha-glucosidase inhibitory and antioxidant activities of *R. mucronata* can be attributed to its phenolic content. Phenolic components have shown effective inhibition of α-glucosidase enzyme as well as antioxidant effect [22-24].

Hence, total phenolic content of *R. mucronata* extracts was determined by Folin-Ciocalteu method. It has been observed that these activities were proportional to phenolic content of *R. mucronata*, as Ethanol extract showed the highest phenolic content (**Table 3**).

TABLE 2: EFFECT OF *R. MUCRONATA* ON DPPH

Extract	Concentration (µg/ml)	% DPPH Scavenged [Mean±SD]	IC_{50} (µg/ml)
Aqueous	10	17.88 ± 5.2	61.48
	40	39.60 ± 4.8	
	60	49.71 ± 3.6	
	80	63.82 ± 1.2	
	100	69.06 ± 3.4	
Ethanol	10	24.31 ± 4.2	34.82
	20	36.09 ± 5.7	
	30	47.31 ± 4.5	
	40	57.26 ± 0.7	
	60	71.77 ± 0.7	
Ascorbic Acid (Standard)	3	24.84 ± 0.9	5.61
	4	34.13 ± 3.4	
	5	43.26 ± 1.7	
	6	54.82 ± 1.9	
	7	63.08 ± 1.8	

TABLE 3: PHENOLIC CONTENT ESTIMATION OF *R. MUCRONATA*

Extract	Gallic acid equivalent (mg/gm)*
Aqueous	340
Ethanol	504

*Mean of Triplicate determinations

Besides, *R. mucronata* has shown anti-HIV activity by inhibiting HIV-reverse transcriptase enzyme and interfering with the gp120/CD4 interaction in our earlier study [25]. It has also revealed putative HIV-protease inhibitory activity [26]. The use of combination antiretroviral therapy consisting of reverse transcriptase and protease inhibitors, has yielded clinical benefits for HIV-infected patients, however, it has also led to adverse metabolic effects such as diabetes [27] and when HIV and diabetes intersect, the treatment regimens required for both diseases can be overwhelming for patients [28]. Thus oxidative stress and anti-HIV drugs are associated with diabetes. Our current and previous studies have shown anti-diabetic, antioxidant and anti-HIV activities of *R. mucronata*. Hence it can be a promising candidate for further investigations.

CONCLUSION: *R. mucronata* can be valuable in treatment of diabetes not only through inhibition of alpha-glucosidase enzyme, but also by its antioxidant effect.

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