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Research Article

IN-VITRO ALPHA AMYLASE INHIBITORY AND ANTI-OXIDANT ACTIVITIES OF ETHANOLIC LEAF EXTRACT OF *CROTON BONPLANDIANUM*

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ABSTRACT

Objective: The objective of the study was to evaluate the α-amylase inhibitory activity against porcine pancreatic amylase (PPA) and antioxidant potential of ethanolic leaf extract of *C.bonplandianum in- vitro.*

Materials and Methods: The leaf extract was prepared with ethanol, filtered, distilled and stored at -20°C. Preliminary phytochemical screening and quantitative estimation of phenols, flavonoids, tannins and total antioxidant capacity along with free radical scavenging activity (DPPH) was carried out. Different concentrations (10,20,40,60,80,100µg/ml) of the extract was subjected to α-amylase inhibitory activity using soluble starch as substrate and the IC_{50} value was calculated. Compounds responsible for these activities were identified by GC-MS with the inbuilt libraries (NIST - 11).

Results: Phytochemical screening revealed the presence of phytoconstituents like phenols, flavonoids, alkaloids, saponins, terpenoids, etc. Presence of polyphenols (41.26±0.39 µg/ml), flavonoids (7.53±0.31 µg/ml) and tannins (46.75±0.14 µg/ml) exhibit good free radical scavenging activity (IC_{50} -170.3±0.30 µg/ml) using DPPH as substrate and total anti-oxidant was found to be 214 ±0.20 µg/ml. The ethanol extract exhibited significant α-amylase inhibitory activity with an IC_{50} value of 17.22 ± 0.05 when compared with acarbose (IC_{50} value 2.65 ± 0.03) ($p<0.001$, n=3).

Conclusion: The ethanol extract exhibit potent α-amylase inhibitory activity which could be attributed to the presence of bioactive constituents like alkaloids, tannins, phenols, saponins, steroids and flavonoids. Thus, it could be concluded that the probable mechanism of action of the extract is due to their inhibitory action on PPA, thereby reducing the rate of starch hydrolysis leading to lowered glucose levels.

Keywords: Croton bonplandianum, polyphenols, flavonoids, α-amylase inhibitory activity.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia with disturbances of carbohydrate, lipid and protein metabolism resulting from defect in insulin secretion, insulin action or both. According to the recent data the prevalence of diabetes is on the rise from 143 million persons to 300 million persons by 2025 [1]. Among various therapeutic approaches to cure diabetes, lowering postprandial hyperglycemia is one such approach. Postprandial hyperglycemia is mainly due to high calorie and nutrition depleted foods leading to the formation of high glycation end products. This plays a role in diabetic complications, cardiovascular disease and aging [2].

One of the effective method to control diabetes is to inhibit the activity of alpha amylase enzyme which is responsible for the breakdown of starch to more simple sugars (dextrin, maltotriose, maltose and glucose) [3]. This is contributed by alpha amylase inhibitors, which delays the glucose absorption rate thereby maintaining the serum blood glucose in hyperglycemic individuals [4]. Some inhibitors in clinical use such as acarbose, miglitol, and voglibose produce serious side effects such as bloating, and abdominal discomfort. Oxidative stress (formation of free radicals) can be generated due to hyperglycemic status through both enzymatic and non-enzymatic processes. These free radicals would damage cellular proteins as well as mitochondrial DNA [5]. Most of the ROS produced are scavenged by endogenous defense system under normoglycemic status. But in diabetes due to hyperglycemic condition the system depend on some exogenous antioxidants from natural resources. Medicinal plants are being used right from ancient times for they are an exemplary source of drug due to its high efficacy, reduced cost and side effect [6]. Phytochemical constituents like saponin, phenols, flavonoids etc studied in various plants such as *P.vulgari, Euphorbia hirta, Cassia glauca* showed potential alpha amylase inhibitors such as sapogenin, Cg-1[7]. The role of medicinal plants in disease prevention is attributed to its antioxidant properties due to their bioactive constituents [8].

MATERIALS AND METHODS

Materials

Porcine pancreatic amylase was purchased from Sigma Aldrich. All the solvents were procured from Merck, India. DPPH, 3, 5-dinitrosalicylic acid (DNSA) and other chemicals were procured from Himedia.

Collection and Processing of the Plant Material

The plant was collected from Chennai, Tamilnadu, India and authenticated, (No:PARC/2011/1021) by Dr. Jayaraman, Director, Plant Anatomy Research Center and deposited in the Department of Biomedical Sciences, Sri Ramachandra University.

Extraction of Leaf Material

100g of the coarsely powdered leaf material was subjected to extraction with 500 ml of ethanol for 72 hours. The extract was filtered through Whatmann filter paper No: 1, distilled and stored at -20°C until use.

Phytochemical Analysis

The extract was analyzed for the active phyto-constituents such as phenols, flavonoids, alkaloids, tannins, saponins, terpenoids etc according to the standard protocol [9].

Quantification of Secondary metabolite

Estimation of Total Phenols

The extract (1mg/ml) was mixed with 20 µl of Folin-Ciocalteau reagent (1:10) and 50 µl of aqueous 2.5% Na_2CO_3. The mixtures were allowed to stand for one hour and determined in spectrophotometer at 765 nm. From the standard graph, the phenol content in the extract was determined using gallic acid as standard [10].

Estimation of Tannin

To the sample, 0.5 ml folins phenol reagent (1:2) and 5 ml of 1% sodium carbonate was added, kept at room temperature (RT) for 5 min and read at 640 nm. From the standard graph, the tannin content in the extract was determined using gallic acid as standard [11].

Estimation of Flavonoid

The extract was treated with 0.1 ml of 10% aluminum chloride and 0.1 ml of 1 M sodium acetate. The absorbance of the reaction mixture was measured at 415 nm after left in RT for 30 min. From the standard graph, the flavonoid content in the extract was determined using quercitin as standard [10].

Anti-oxidant activity of the leaf extract

DPPH Radical Scavenging Activity

About 10 µL (2-1000 µg/ml) of test sample solution was added to 190 µL DPPH (150 µM) in ethanol solution and incubated for 30 minutes at 37°C. The control contains DPPH without extract. The decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was measured at 517 nm and the percentage inhibition was calculated. The IC_{50} value was determined as the concentration of the test mixture that gave 50% reduction in the absorbance from a control [12].

Total anti-oxidant activity

An aliquot of 100 µl extract was combined with reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) and boiled at 95ºC for 60-90 min and the absorbance was measured at 695 nm [13].

***In –Vitro* Alpha-amylase inhibitory assay**

To 600 µl of (10,20,40,60,80,100µg/ml) plant extract, 1.2 ml of starch in phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride was added. The reaction was initiated by adding 600 µl porcine pancreatic amylase and incubated at 37ºc. From the above mixture 600 µl was taken and 300 µl of DNSA (1g of DNSA, 30g of sodium potassium tartarate and 20 mL of 2N sodium hydroxide was added and made up to a final volume of 100 mL with distilled water) and kept it in a boiling water bath for 15 minutes. The reaction mixture diluted with 2.7 ml of water and absorbance was read at 540 nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 600 µL in distilled water. Control, representing 100% enzyme activity was prepared in a similar manner, without extract. The experiments were repeated thrice using the same protocol [14].

Method for calculation of α-amylase inhibitory activity

The α-amylase inhibitory activity was calculated by using the formula:

The α-amylase inhibitory activity = (Ac+) – (Ac-)- (As-Ab) / (Ac+)-(Ac-) x 100

where, Ac+, Ac-, As, Ab are defined as the absorbance of 100% enzyme activity (only solvent with enzyme), 0% enzyme activity (only solvent without enzyme), a test sample (with enzyme) and a blank (a test sample without enzyme) respectively [15].

Statistical analysis

All measurements were carried out in triplicates. Statistical analysis was done with SPSS software (version 17.0). Mean ± SD was used for multivariate analysis (ANOVA) with Twkey's Post hoc test. Difference at $p < 0.05$ was considered statistically significant.

GC-MS analysis

GC-MS analysis was carried out on GC-MS-5975C [AGILENT] under the following conditions. Helium was used as the carrier gas at a constant flow rate of 1.51 ml/min and 2 µl of sample was injected. The column temperature was programmed to 70ºC with increasing temperature of 10ºC/min to 300ºC. The mass spectra were obtained through ionization energy of 70 eV in the EI mode. Total GC- MS running time was 30 min. The organic compounds were identified by comparison of mass spectra with the inbuilt libraries (NIST - 11).

RESULTS

The results showed that the extract exhibited dose dependent alpha amylase inhibitory activities by *in vitro* assay using starch as substrate and good anti-oxidant property.

Phytochemical screening and Quantification of Secondary Metabolite

Plant derived compounds are well known for their therapeutic values since ancient times. The qualitative analysis of the ethanol extract confirms the presence of phenols, flavonoids, tannins, steroids, alkaloids, saponins and terpenoids (Table 1). Quantitative estimation of secondary metabolites (Table 2) was found to be phenols (41.26±0.39 µg/ml), flavonoids (7.53±0.31 µg/ml) and tannins (46.75±0.14 µg/ml).

Table 1: Phytochemical screening of ethanolic leaf extract

S.no	Phytochemicals	EtE
1	Phenol	+++
2	Flavonoids	++
3	Tannin	+++
4	Alkaloids	++
5	Steroids	++
6	Terpenoids	++
7	Saponins	++
8	Antraquinones	-

+ - Present ; - Absent ; EtE- ethanol extract

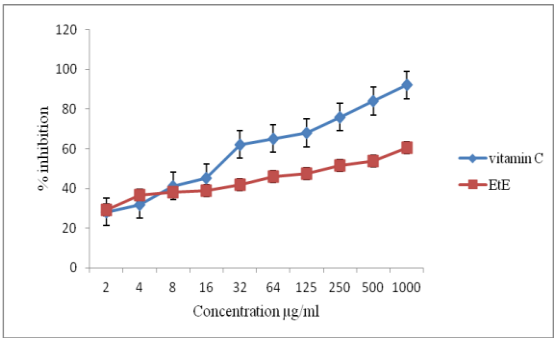
Table 2: Secondary metabolite content in the leaf extract

S.no	Secondary metabolite	EtE (µg/ml)
1	Phenols[1]	41.26±0.39
2	Flavonoids[2]	7.53±0.31
3	Tannins[3]	46.75±0.14

Each value was expressed in Mean ± SME (n=3) [1, 3] µg gallic acid equivalent ; [2] µg quercitin equivalent

DPPH and Total anti-oxidant activity

Ethanol extract shows potent concentration dependent scavenging activity on DPPH (IC_{50} -170.3±0.3 µg/ml). Ascorbic acid (IC_{50} -19.36 ±0.3 µg/ml) was used as a standard (Graph 1). Total anti-oxidant activity of the leaf extract was evaluated by the phosphomolybdenum method by reducing the Mo (VI) Mo (V) complex at acidic pH. Ethanol extract (214 ±0.2 µg of tocopherol/mg of extract) showed good anti-oxidant activity could be correlated with high phenolic content.



Graph 1: DPPH radical scavenging activity of ethanolic extract

***In-vitro* alpha amylase inhibitory assay**

Acarbose is a standard drug for alpha amylase inhibitor. Acarbose at a concentration of (10-100µg/ml) showed alpha amylase inhibitory activity from 55.92 ±0.31to 93.61±0.56% with an IC_{50} value 2.65 µg/ml (Table 3). Ethanolic extract (10-100µg/ml) of *C. bonplandianum* exhibited potent alpha amylase inhibitory activity in a dose dependent manner. Ethanol extract showed highest inhibitory activity from 38.11 ± 0.37 to 83.60 ± 0.09 with an IC_{50} value of 17.22 µg/ml.

Table 3: α-amylase inhibitory effects of *C.bonplandianum* leaf extract in comparison with standard drug acarbose

S.no	Plant extract	Concentration (µg/ml)	% of inhibition	IC_{50} value µg/ml
1	Ethanol extract of *C.bonplandianum*	10	38.11 ± 0.37	17.22 ± 0.05***
		20	53.80 ± 0.31	
		40	59.66 ± 0.64	
		60	66.59 ± 0.52	
		80	72.10 ± 0.07	
		100	83.60 ± 0.09	
2	Acarbose	10	55.92 ± 0.31	2.65 ± 0.03
		20	65.73 ± 0.56	
		40	73.83 ± 0.73	
		60	82.55 ± 0.77	
		80	82.86 ± 0.49	
		100	93.61 ± 0.56	

***** p < 0.001 ; n=3**

GC-MS analysis

The GC-MS study reveals the presence of many phytocompounds which contribute to the antioxidant and α- amylase inhibitory activity (Figure 1). The main compounds Phenol, 2,4-bis(1,1-dimethylethyl) (22.31%), 5-Eicosene (12.99%), Cyclotetracosane (2.62%) Asparagine N-dl-alanyl (1.29%), Phytol (2.34%) are identified (Table 4).

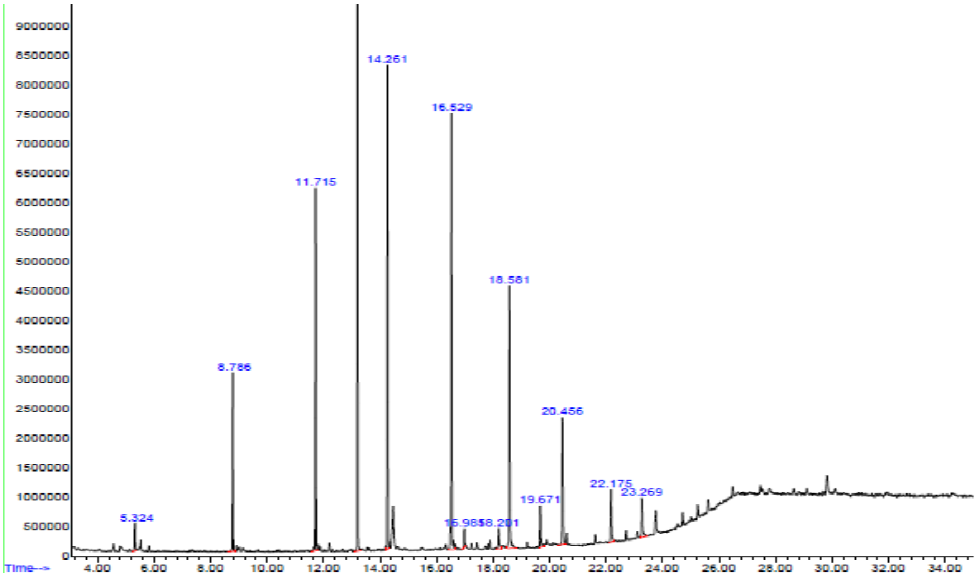


Figure1: Chromatogram of ethanolic extract *C.bonplandianum* by GC-MS

Table 4: Phytocomponents identified in the ethanolic leaf extract of *C.bonplandianum* by GC-MS.

S.no	Name of the compound	Molecular weight	Retention time (min)	Area %
1	Cyclopentylcyclohexane	152.27	16.986	0.79
2	3,7,11-Trimethyl-2,4-dodecadiene	208.38	16.986	0.79
3	Asparagine, N-dl-alanyl	246.26	18.205	1.29
4	1-Dodecanamine	185.34	18.205	1.29
5	Tetraacetyl-d-xylonic nitrile	343.28	18.205	1.29
6	5-Eicosene	280.53	18.583	12.99
7	E-15-Heptadecenal	252.43	18.583	12.99
8	Phytol	296.53	19.672	2.34
9	3-Fluorobenzoic acid	140.11	19.672	2.34
10	2-Propenamide	127.18	19.672	2.34
11	5-Nonadecene	266.5	20.457	6.81
12	Behenic alcohol	326.6	20.457	6.81
13	Phenol, 2,4-bis(1,1-dimethylethyl)	206.32	13.195	22.31

14	Cyclotetracosane	336.63	22.17	2.62
15	Hexacosyl acetate	424.43	22.17	2.62
16	Phthalic acid	166.13	23.274	2.4

DISCUSSION

Herbal plants have long been used to treat diabetes, as their principal bioactive components showed good anti-diabetic and anti-oxidant properties [16]. Many herbal plant extracts have been reported for their alpha amylase inhibitory activity but to date no such report was found for *C.bonplandianum*. Our study is the first report to state that the ethanolic extract of the leaves, showed potent alpha amylase inhibitory activity indicating the presence of potential inhibitors such as Tannins, phenols, flavonoids etc. These alpha amylase inhibitors are also called as starch blockers since it prevents or slows the absorption of starch in to the body mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltriose and other simple sugars [4]. In our study, the ethanol extract showed maximum α- amylase inhibitory activity (IC_{50} 17.22 µg/ml) which could be attributed to the presence of polyphenols (41.26±0.39 µg/ml) and flavonoids (7.53±0.31) because polyphenols are not only capable of reducing oxidative stress but also of inhibiting carbohydrate hydrolyzing enzymes because of their ability to bind with proteins [17,18]. Our results are in accordance with the previous study wherein, there is a positive relationship between the total polyphenol and flavonoid content and the ability to inhibit intestinal α-glucosidase and pancreatic α-amylase [19, 20].

In our study acarbose was used as the positive control; it inhibited the α-amylase activity with an IC_{50} value 2.65 µg/ml while the IC_{50} value of the extract was found to be 17.22 ± 0.05 µg/ml. This indicates that the ethanolic extract is very potent α-amylase inhibitor in comparison with acarbose. This could be justified that the nature of some extract constituents (phenols, flavonoids saponins, steroids, alkaloids, terpenoids) present in the extract could be responsible as being effective inhibitors of α-amylase [21].

Oxidative stress plays a pivotal role in the development of diabetes complications. Free radicals are formed disproportionately during diabetes due to glucose oxidation and the subsequent oxidative degradation of glycated proteins [22]. In addition, the diabetic patients have enhanced cellular oxidative stress and reduced antioxidant potential leads to defective antioxidant status. The present study also involved the evaluation of antioxidant capacity which showed good DPPH (170.3±0.3 µg/ml) scavenging activity and total antioxidant capacity (214 ±0.2 µg of tocopherol/mg of extract).

Although the presence of flavonoids and polyphenolics could contribute to hypoglycemic activity, the specific bioactive compounds which are responsible for these activities were studied through GC-MS analysis. This study revealed the presence of 17 compounds whose biological activities have been reported earlier. Phytol is found to have antimicrobial, anti-oxidative and anti-diabetic property [23]. E-15 Heptadecanol, 1-Nonadecene, 5-Eicosene, Asparagine, 2-Tetradecene have also proven to show antioxidant and anti-diabetic effects [24,25]. Phenol, 2,4-bis (1,1-dimethylethyl) [26] and 3-Fluorobenzoic acid was reported to have antioxidant activity [27]. Cyclotetracosane has already been reported for their α-amylase inhibitory activity [28].

CONCLUSION

The study result indicates that *C.bonplandianum* exhibit potent α-amylase inhibitory activity and this therapeutic potentiality could be exploited in the management of post prandial hyperglycemia in treatment of Type 2 diabetes mellitus. Further, this study directs future research in separating the bioactive compound responsible for this activity.

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Conflict of Interest

None declared conflict of interest.

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