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Original Article

In vitro alpha amylase inhibitory and antioxidant activity studies on the root of *Chonemorpha fragrans* (Moon) Alston

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ABSTRACT

Traditional therapy of diabetes includes the use of variety of plant derived products. Among these only a few alpha amylase inhibitors were explored. In this investigation, the *in vitro* alpha amylase inhibitory activity and the antioxidant potential of total ethanolic as well as petroleum ether, chloroform, ethyl acetate and residual alcoholic fractions of the root of *C. fragrans* were studied. The chloroform and ethyl acetate extracts showed appreciable alpha amylase inhibitory activity and the IC_{50} values were found to be 42.5 and 47.5 µg/ml respectively, where as the standard acarbose exhibited an IC_{50} value of 25 µg/ml. The strong antioxidant properties were revealed with different models like DPPH, ABTS, Ferric reduction, nitric oxide and total antioxidant models.

Keywords: Diabetes, alpha amylase, *Chonemorpha fragrans*, acarbose, free radical scavenging.

INTRODUCTION

Diabetes mellitus is a complex syndrome involving severe insulin dysfunction in conjunction with gross abnormalities in glucose homeostasis and lipid metabolism. The disease is categorised into two major subgroups. One group is insulin dependent diabetes mellitus (IDDM) or Type I and second category is non-insulin dependent *Diabetes mellitus* (NIDDM) or Type II. IDDM diabetes is most evident in the young people whereas NIDDM is strongly age related. The frequency of the disease is increasing day by day and according to the WHO report, 300 million people are likely to be affected by 2025 and India is projected to have the largest diabetic cases [1].

Type 2 diabetes is one of the primary threats to human health due to increasing prevalence, chronic course and disabling complications. Many diverse therapeutic strategies for the treatment of Type 2 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 hypoglycaemic agents such as biguanides and sulphonylureas and the inhibition of degradation of dietary starch by glycosidases such as α-amylase and α-glucosidase [2]. The high prevalence of diabetes and its long term complications have led to an ongoing search for active principles form natural sources.

Chonemorpha fragrans is a traditional plant distributed throughout the moist forests of India, Malaya, Indochina and Philippines. It is a giant stout climber with large, sweet-scented, flowers. It was used in many diseases including diabetes, jaundice, cough, bronchitis and intermittent fever. Chonemorphine a steroidal alkaloidal substance isolated from the roots were found to possess antiamoebic property. Roots were also found to be antidiabetic in alloxan induced rat models. Camptothecin was isolated from the callus cultures of *C. fragrans*. Other phytoconstituents isolated from this plant include japindine, N-formylchonemorphine, funtumafrine-C, bauerenol acetate, bita-sitosterol, taraxasterol, lipids such as glycerides of lauric, stearic, arachidic, myristic, palmitic, oleic,linolenic and recinoleic acid[3-4].

Inhibition of enzymes like alpha amylase will reduce the postprandial hyperglycaemia which is useful in the control of diabetes. So the present study was undertaken to evaluate the alpha amylase inhibitory potential of the total ethanolic extract and the different fractions of the roots of *C.*

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fragrans. Since oxidative stress and free radicals are implicated in conditions like diabetes, so the antioxidant potential of the total ethanolic extract was also evaluated.

MATERIALS AND METHODS

Plant material: Roots of *C. fragrans* were collected during the month of September 2010 from Thengamam, Kerala. The plant material was authenticated by Mr. Rojimon P.Thomas, Department of Botany, CMS College, Kottayam and a voucher specimen No.CMS-272 was deposited in the department.

Preparation of the plant extract: Shade dried roots (2 kg) were powdered and extracted with ethanol (95%) by soaking them overnight and refluxing for 3 hours and the procedure was repeated thrice. The combined extract was concentrated to a semisolid mass(yield :4%w/w).The total ethanolic extract was fractionated with petroleum ether, chloroform, ethyl acetate and the remaining was marked as residual alcoholic fraction.

EXPERIMENTAL DESIGN

DPPH radical scavenging assay [5]: DPPH scavenging activity was measured by the spectrophotometric method. To a methanolic solution of DPPH (200µM), 0.05ml of the test compounds dissolved in methanol were added at different concentration (2-1000µg/ml). An equal amount of methanol was added to the control. After 20 minutes, the decrease in the absorbance of the test mixture was read at 517 nm and the percentage inhibition was calculated using the formula

$$\%\ Inhibition = \frac{(control - test)}{control} \times 100$$

ABTS radical cation decolourisation assay [5]: A 2mM solution of ABTS was mixed with 0.17mM potassium persulfate prepared in distilled water. 0.3 ml of 17mM potassium persulfate was added to 50 ml ABTS solution. The mixture was covered with an aluminium foil and stored overnight in dark at room temperature before use. Different concentrations of test drug and standard, ascorbic acid (1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/ml) were prepared. Different concentrations of fractions and standard were added to the ABTS solution and the absorbance was measured at 734nm.

***Scavenging of Nitric oxide radical [5]*:** Nitric oxide was generated from sodium nitroprusside and measured by Griess'reaction. Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside ($5mmolL^{-1}$) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract (250-1000µg mL^{-1}) prepared in methanol and incubated at 25° for 30minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride).

Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathylethylene diaminedihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard ascorbic acid.

% inhibition =

$$\frac{\text{control}-\text{test}}{\text{control}} X100$$

Total antioxidant Capacity [6]: The assay is based on the reduction of molybdate-IV (Mo^{IV}) to molybdate-V (Mo^{V}) by the extracts and subsequent formation of green phosphate/Mo^{V} complex in acidic pH. 0.1 ml of the extract (10mg/ml) dissolved in 10% DMSO was mixed with 1ml of the reagent(0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate), incubated at 95° for 90 min, cooled to room temperature and absorbance was measured at 695nm against a blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid using standard plot.

***Orthophenanthroline method [7]*:** The reaction mixture of 1ml orthophenanthroline(0.005g in 10ml methanol), 2ml ferric chloride 200µM (3.24 mg in 100ml distilled water) and 2ml of various concentrations of the fractions were incubated at ambient temperature for 10 min and its absorbance was measured at 510 nm against a reagent blank. Control was measured omitting the sample.

***In vitro α amylase inhibitory assay [8]*:** 25µl of 10 mg/ml of the extract and 25µl of 25mM phosphate buffer pH 6.9, containing porcine α amylase at a concentration of 0.5 mg/ml were incubated at 25° for 10 min. After pre incubation, 25µl of 0.5% starch solution in 25mM phosphate buffer pH 6.9 was added. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 50µl of 96mM 3, 5-dinitrosalicylic acid colour reagent. The micro plate was then incubated in a boiling water bath for 5 min and cooled to room temperature. Absorbance was

measured at 540nm using a micro plate reader. The percentage inhibition was calculated as follows

% inhibition = $\frac{\text{control}-\text{test}}{\text{control}} \times 100$

Control inhibitions represent 100% enzyme activity and were conducted in a similar way by replacing extracts with vehicle.

Statistical analysis: All the experiments were done in triplicate. Values are expressed as mean±SEM.

RESULTS

The IC_{50}values obtained from different antioxidant models were calculated and given in table No.1.
The alpha amylase inhibitory activity shown by the total ethanolic and various fractions of C. fragrans at different concentrations are given in table No. 2 and the IC_{50}values are shown in figure No.1.

DISCUSSION

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radicals can bring about thousands of reactions and thus cause extensive tissue damage. Lipids, Proteins and DNA are all susceptible to attack by free radicals [9]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals.

Oxidative stress is currently suggested as the mechanism underlying diabetes and diabetic complications. Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental *Diabetes mellitus* are thought to be the aetiology of chronic diabetic complications. Several concentrations of the total ethanolic extract of *C. fragrans* (2-1000µg/ml) were hence subjected to different *in vitro* antioxidant studies *viz* total antioxidant, ABTS, DPPH, nitric oxide scavenging and ferric reduction by orthophenanthroline method.

The extract was observed to scavenge free radicals in a concentration dependant manner in all the models tested. From the results IC_{50} values were found to be 36.0µg/ml,110.0µg/ml, 51.5µg/ml and 497.0µg/ml for DPPH, ABTS, ferric reduction and nitric oxide scavenging models respectively. This suggests that the ethanolic extract of *C. fragrans* contains substantial amounts of antioxidants, which may have an important role in the improvement of disorders involving oxidative stress in diabetes mellitus.

Control of postprandial plasma glucose levels is critical in the early treatment of *D. mellitus* and in reducing chronic vascular complications. One of the therapeutic approaches for reducing postprandial hyperglycemia in patients with DM is to prevent absorption of carbohydrates after food uptake. Only monosaccharaides, such as glucose and fructose can be transported out of the intestinal lumen into the blood steam. Complex starches, oligosaccharides and disaccharides must be broken down into individual monosaccharaides before being absorbed in the duodenum and upper jejunum. This digestion is facilitated by enteric enzymes, including pancreatic α-amylase and α-glucosidase that are attached to the brush border of the intestinal cells [10].

Alpha amylase inhibitors that reduce postprandial hyperglycaemia by suppressing hydrolysis of starch have been found useful in the control of diabetes. Many of the herbal extracts have been reported to possess antidiabetic activity and are currently being used for the treatment of diabetes. But the exact mechanism by which they act were not studied. In the present study the total ethanolic extract and the petroleum ether fraction, chloroform fraction, ethyl acetate fraction and the residual alcoholic fractions of *C. fragrans* root were investigated for their alpha amylase potential.

The total ethanolic extract showed 50% inhibition at a concentration of 135 µg/ml. The petroleum ether, chloroform extract, ethyl acetate and residual alcoholic extracts showed 50 % inhibition at a concentration of 287.5, 42.5, 47.5, 197.5 µg/ml respectively. These results shows that the Chloroform extract exhibited the maximum activity followed by ethylacetate fraction and the results are comparable with that of the standard acarbose which showed 50% inhibition at a concentration of 25µg/ml.

From the above results, it was concluded that the *C. fragrans* root extract is a rich source of natural antioxidants which might be useful in preventing the progress of various oxidative stress. The activity shown by the chloroform extract demonstrates the potential of this extract in the development of future antidiabetic candidates. Further studies are in progress to isolate the phytoconstituents responsible for the bioactivity of the plant.

CONCLUSION

The results suggests that the ethanolic extract of roots of *C. fragrans* showed a potent *in vitro* antioxidant activity and the percentage inhibitions were comparable to that of standard ascorbic acid. The α-amylase inhibitory studies on the total ethanolic and various fractions showed that the

chloroform extract exhibited maximum activity when compared to the standard acarbose. So the present study demonstrates the potential of *C. fragrans* as a candidate for the development of phytoconstituents in the treatment of diabetes. These findings also suggests that the inhibition of alpha amylase may be one of the mechanisms by which *C. fragrans* exhibits its antidiabetic activity.

Table 1. Comparison of ic_{50} values of ethanolic extract with standard ascorbic acid

Sl No	**Model**	**IC_{50} value of Total Ethanolic extract (µg /ml)**	**IC_{50} value of Ascorbic Acid standard (µg /ml)**
1	DPPH Scavenging	36.0	3.3
2	ABTS Scavenging	110.0	49.0
3	Ferric reduction	51.5	3.5
4	Nitric oxide scavenging	497	38.68
5	Total antioxidant activity	10mg/ml ethanolic extract of *C. frgrans* is equivalent to 457µg/ml of ascorbic acid	

All the experiments were done in triplicate.

Table 2. Alpha amylase inhibitory activity of different extracts of *C. fragrans* at different concentrations

Sl No	**Extract**	**Average % Inhibitions at Concentrations (µg/ml)**				
		12.5	**25**	**50**	**100**	**200**
1	Total ethanolic extract	8.33±1.36	27.43±1.41	35.30±1.64	41.87±2.08	52.50±2.48
2	Petroleum ether fraction	6.72±1.57	24.03±1.82	26.56±1.02	32.74±1.52	40.32±1.71
3	Chloroform fraction	41.26±1.96	45.45±1.60	53.94±1.85	61.20±1.20	75.87±1.88
4	Ethyl acetate fraction	9.82±1.28	22.80±1.68	54.91±1.70	56.191±1.55	64.68±1.60
5	Residual alcoholic fraction	13.08±1.02	26.22±0.97	38.66±0.88	46.73±0.94	51.71±0.91
6	Acarbose	45.09±0.78	66.34±1.38	74.24±0.83	80.79±0.86	83.06±0.37

All the experiments were done in triplicate. Results are expressed as Mean±S.E.M

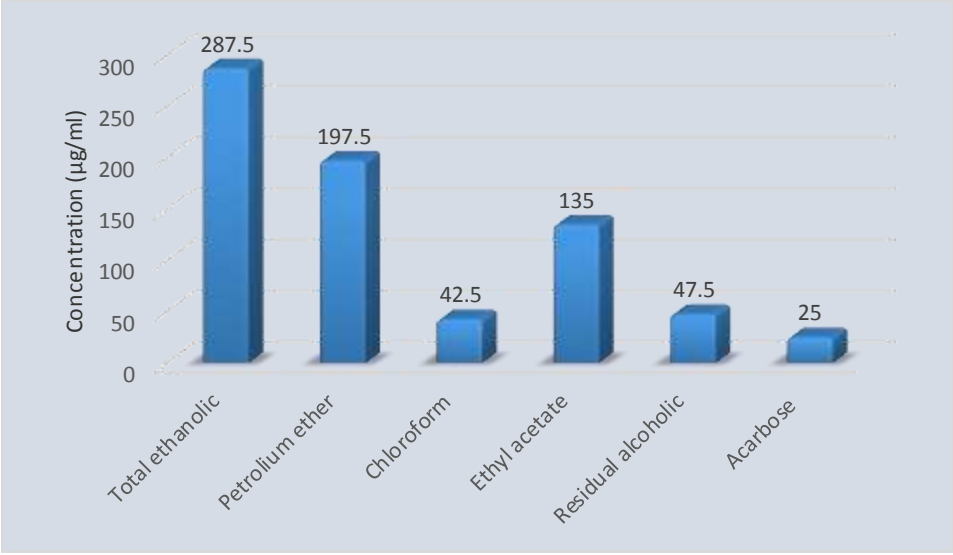

Figure 1. IC_{50} values of total ethanolic extract and different fractions of *C. fragrans*

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