Original Article

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IN VITRO ANTI-OXIDANT AND ALPHA-AMYLASE INHIBITORY ACTIVITY OF ISOLATED COMPOUND FROM ETHYL ACETATE EXTRACT OF *CYNODON DACTYLON* AND *PIPER BETLE*

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Abstract

In vitro methods play important role for the preclinical studies for any activity, which makes support to the in vivo studies. *Cynodon dactylon* (L.) Pers (Gramineae family), possesses various medicinal property, Piper betle belongs to the family piparaceae. In the South East Asia region, *Piper betle L*. are among the plants that have been associated with the control of caries and periodontal diseases. The dried materials were coarsely powdered using an electric blender. Powdered materials (500g) were then packed in soxhlet apparatus and successively extracted with petroleum ether, Benzene, chloroform, ethyl acetate, methanol and water. Each time before extraction with the next solvent, the powdered materials were dried in hot air oven at below 50°C. About 2 gms of the concentrated ethyl acetate extract was mixed with suitable quantity of silica gel (100-200 mesh). The elute was concentrated by evaporating the solvent and the residues named as CD isolate (90% of Chloroform: 10 % of Ethyl acetate) and PB isolate (50% of Toluene: 50 % of Ethyl acetate) were identified by High performance Thin Layer Chromatography. The selected isolates from Cynodon dactylon and Piper betle and the combination of both were found to have antioxidant activity in different models in the present study. The antioxidant activity of these isolates might be due to inactivation of free radical or complex formation with metal ion, or a combination of both. α-Amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The result indicate that the selected isolate posses better antioxidant and alpha amylase inhibitory activity in combined form when compare to individual form.

Key words: Cynodon dactylon, Piper betle, Anti-oxidant activity, Alpha amylase inhibitory activity.

Introduction

The marketable development of plants as source of antioxidants to augment health and food defense is of current deliberation. Epidemiological studies have optional positive associations between the utilization of phenol-rich foods or beverages and the prevention of diseases[1]. Free radicals are fashioned when cells use oxygen to engender energy.

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These by-products are normally reactive oxygen species (ROS) such as super oxide anion, hydroxyl radical and hydrogen peroxide that consequence from the cellular redox course[2]. Diabetes Mellitus, a metabolic chaos of numerous etiologies, is characterized by chronic hyperglycemia with strife of carbohydrate, fat and protein metabolism that consequences from imperfections in insulin secretion, insulin action or both. Previously comparison of various extract of *Cynodon dactylon* and *Piper betle* was reported for antioxidant activity. *Cynodon dactylon* (L.) Pers (Gramineae family), generally known as "Njem" in Morocco, possesses various medicinal property[3,4]. Piper betle belongs to the family piparaceae. Over

700 species of plants are belonging to piper. In the South East Asia region, *Piper betle L*. are among the plants that have been associated with the control of caries and periodontal diseases[5,6,7]. In-vitro methods play important role for the preclinical studies for any activity, which makes support to the in-vivo studies. The present work was planned to evaluate the effect of isolated fractions on oxidative stress events by antioxidant and α-amylase inhibition in in-vitro pharmacological models.

Materials and Methods

The plant materials such as Cynodon dactylon and Piper betle were collected from Local market in that Cynodon dactylon were collected from Departmental medicinal garden of Annamacharya college of Pharmacy, Rajampet, Andhrapradesh. The collected plant materials were identified and authentified by Dr. Madhava chetty, Department of Botany, Sri Venkateswara University, Tirupati.

Preparation of extract

The freshly collected plant materials were washed; shadow dried and then dried in hot air oven at a temperature not more than 50°C. The dried materials were coarsely powdered using an electric blender. Powdered materials (500g) were then packed in soxhlet apparatus and successively extracted with petroleum ether, Benzene, chloroform, ethyl acetate, methanol and water. Each time before extraction with the next solvent, the powdered materials were dried in hot air oven at below 50°C. Finally extracts were concentrated in rotary evaporator at a temperature not more than 50°C and then, dried under vacuum desiccators. The dried extracts thus obtained were used for Isolation[8].

Isolation

About 2 gms of the concentrated ethyl acetate extract was mixed with suitable quantity of silica gel (100-200 mesh) to ensure the free flow of the extract along with adsorbent it was packed in the column through the funnel, then petroleum ether was added through the column and kept aside over night. Then the column was eluted with different organic solvents. The fraction 100ml each of the elute from the column was collected into series of 500ml glass beakers. The elute was concentrated by evaporating the solvent and the residues named as CD isolate (90% of Chloroform: 10 % of Ethyl acetate) and PB isolate (50% of Toluene: 50 % of Ethyl acetate) were identified by High performance Thin Layer Chromatography.

Anti-oxidant activity

Free Radical Scavenging Activity

Different concentrations (10µg, 50µg and 100µg) of sample and Butylated hydroxy anisole (BHA – synthetic antioxidant) were taken in different test tubes. The volume was adjusted to 500µl by adding Methanol. Five milliliters of a 0.1 mM methanolic solution of 1,1-diphenyl-2-picryl hydrazyl (DPPH) was added to these tubes and shaken vigorously. A control without the test compound, but with an equivalent amount of methanol was maintained. The tubes were allowed to stand at RT for 20 min.[9,10,11] The absorbance of the samples was measured at 517 nm. Radical scavenging activity was calculated using the following formula,

$$\text{Percentage radical scavenging activity} = \frac{(\text{control Abs} - \text{sample abs}) \times 100}{\text{Control abs}}$$

Hydroxyl Radical Scavenging Activity

Various concentrations (10µg, 50µg and 100µg) of sample were taken in different test tubes and made up to 250µl with 0.1M phosphate buffer. Ascorbic acid (AA) was used as standard for comparison. One milliliter of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA (0.018%), and 1 ml of Dimethyl sulphoxide (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. These reaction mixtures were incubated at room temperature for 15 min. The reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (150 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for color development. The yellow color formed intensity was measured spectrophotometrically at 412 nm against reagent blank. The percentage hydroxyl radical scavenging activity was calculated by the following formula,

$$\% \text{ hydroxyl radical scavenging activity} = 1 - \frac{\text{Difference in absorbance of sample} \times 100.}{\text{Difference in absorbance of blank}}$$

Ferric Reducing Antioxidant Power

Various concentrations of sample (10µg, 50µg and 100µg) were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Next, 2.5 mL of 10% (w/v) trichloroacetic acid was added. 5 mL of above solution was mixed with 5 mL of distilled water and 1 mL of 0.1% of ferric chloride. The absorbance was measured spectrophotometrically at 700 nm. Butylated hydroxy anisole (BHA) was used as standard antioxidant.

Nitric oxide radical Scavenging Activity

Various concentrations (10µg, 50µg and 100µg) of sample and Butylated hydroxy anisole (BHA) were taken in different test tubes and made up to 3ml with 0.1M phosphate buffer (pH 7.2). Sodium Nitroprusside (5mM) prepared in buffered saline (pH7.2) was added (1 ml) to each tube. The reaction mixture was incubated for 30 min at RT. A control without the test compound was maintained. After 30 min, 1.5 ml of above solution was mixed with 1.5 ml of Griess reagent (1% Sulphanilamide, 2% phosphoric acid and 0.1% N-1- Naphthylethylenediamine dihydrochloride). The absorbance of the samples was measured at 546 nm. Nitric oxide radical scavenging activity was calculated using the following formula,

$$\% \text{ NO radical scavenging activity} = \frac{(\text{control OD} - \text{sample OD}) \times 100.}{\text{Control OD}}$$

Alpha-Amylase Inhibitory Activity

The activity of amylase (Himedia, Mumbai) was assayed with different concentrations of sample, with control. The test tubes contained 2 ml reaction mixture with sodium phosphate buffer (50 mM, pH 7.0-7.3), different volumes of sample, starch (in buffer) and enzyme (from 1mg/ml sample in buffer). Concentrations screened: 100, 50, 25, and 10 µg for PB Isolate and 100, 200, 500 and 100 µg for CD Isolate and PB and CD Isolate. For every different concentration of sample analyzed, a different blank was maintained. The blank tubes were added with 1 ml of DNS before adding enzyme.[12] The tubes were incubated at 370C for 10 min followed by addition of 1 ml of DNS. The tubes were incubated in boiling water bath for 10 min, cooled and read for absorbance at 540 nm against blank. The maltose liberated was determined by the help of standard maltose curve and activities were calculated according to the following formula,

$$\text{Activity} = \frac{\text{Conc. of Maltose liberated X ml of enzyme used}}{\text{Mol. wt of maltose X incubation time (min)}} \text{ X dilution factor}$$

The inhibitory/induction property shown by the sample was compared with that of control and expressed as percent induction/inhibition. This is calculated according to the following formula,

$$\% \text{ inhibition/induction} = \frac{\text{Activity in presence of compound}}{\text{Control Activity}} \text{X } 100$$

Results

All the results were represented in the form of tables, graphs and the data was subjected for Anti-oxidant and alpha amylase inhibitory activity of combined and individual isolates from *Cynodon dactylon* and *Piper betle*.

Table 1: Percentage free radical scavenging activity

concentration	PB Isolate	CD Isolate	PB + CD Isolate	BHA
20µg	12.28	22.79	24.21	59.3
100 µg	12.28	22.79	24.21	87.08
200 µg	12.28	22.79	24.21	*

Table 2: The Percentage Hydroxyl radical scavenging activity

Concentration	PB Isolate	CD Isolate	PB + CD Isolate	Ascorbic acid
20µg	52.11	55.12	56.02	46.99
100 µg	56.02	59.04	59.34	54.52
200 µg	60.24	61.75	61.14	70.18

Table 3: Ferric reducing anti-oxidant power

Concentration	PB Isolate	CD Isolate	PB + CD Isolate	BHA
10µg	0.011	0.009	0.10	0.11
50 µg	0.014	0.016	0.297	0.303
100 µg	0.021	0.028	0.520	0.546

Table 4: Nitric oxide radical scavenging activity

Concentration	PB Isolate	CD Isolate	PB + CD Isolate	BHA
10µg	3.55	1.88	3.1	3.8
50 µg	7.10	4.11	9.2	10.2
100 µg	11.83	6.21	17.8	19.8

Table 5: Alpha amylase inhibitory activity

Sample	OD at 540 nm	Cocn. of Maltose liberated (µg)	Activity (µmoles/ml/min)	% activity
Control	0.54	43	0.011	100
PB (100 µg)	0.31	24	0.006	54.55
PB (50 µg)	0.47	37	0.0102	92.73
PB (25 µg)	0.49	39	0.0108	98.18
PB (10 µg)	0.53	41	0.014	127.27
CD (100 µg)	0.62	49	0.013	118.18
CD (200µg)	0.54	43	0.011	100.00
CD (500µg)	0.39	30	0.009	81.82
CD (1000 µg)	0.28	21	0.005	45.45
PB+CD (100 µg)	0.73	58	0.016	145.45
PB+CD (200 µg)	0.42	33	0.009	81.82
PB+CD (500 µg)	0.24	18	0.0049	44.55
PB+CD (1000 µg)	0.23	17	0.0047	42.73

PB – Isolated compound from *Piper betle*
CD – Isolated compound from *Cynodon dactylon*

Discussion

The selected isolates from Cynodon dactylon and Piper betle and the combination of both were found to have antioxidant activity in different models in the present study. On comparison, it was found that combination of two isolates has the highest antioxidant activity. The antioxidant activity of these isolates might be due to inactivation of free radical or complex formation with metal ion, or a combination of both. In biochemical system, superoxide radical and H_2O_2 react together to form a single oxygen and hydroxyl radical, this can attack and destroy almost all known biochemical[13]. α-Amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity, in the digestive tract of humans, is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes[14]. This work suggesting that the plant contained lipophilic, potential α -amylase inhibitor and Antioxidant compounds which may contribute to its in vitro antidiabetic effect. It is first to report a potential mode of action of isolates from Cynodon dactylon in combination with Piper betle and suggests that the glucose lowering effect of this plant is due, at least in part, to the inhibition of α –amylase.

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