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Invitro assessment of free radical scavenging activity of *Cynodon dactylon*

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ABSTRACT

The present study was designed to investigate the invitro antioxidant activity of the hydro alcoholic extract of aerial parts of Cynodon dactylon (Family: Poaceae) which is used in folklore medicine for the treatment of various diseases. In vitro scavenging activity was assessed by different methods viz. DPPH radical scavenging activity, superoxide anion radical scavenging assay, nitric oxide scavenging assay, Ferrous chelating ability, hydroxyl radical scavenging assay, hydrogen peroxide scavenging activity and ABTS assay. In all the methods, the extract showed its ability to scavenge free radicals in a concentration dependant manner. Its antioxidant activity was estimated by IC_{50} *values. Superoxide anion radical scavenging assay showed a maximum inhibition of 93.33%. The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic and flavonoid compounds present in the extract. Total Antioxidant capacity of the extract was measured using phosphomolybdenum method. Total antioxidant capacity equivalent of ascorbic acid was 172.39 mg/g of extract. The results obtained from this study reveal that the hydro alcoholic extract of Cynodon dactylon is rich in antioxidant components with several mechanisms of eliciting antioxidant actions which provide scientific basis for its use in folk medicine.*

Key words: *Cynodon dactylon*, DPPH, ABTS, antioxidant.

INTRODUCTION

Oxygen is essential for the aerobic process; cells under aerobic conditions are threatened with the insult of reactive oxygen metabolites, a threat which is efficiently taken care of by the powerful antioxidant system in the human body. Aerobic life is characterized as the continuous production of free radicals balanced by an equivalent synthesis of antioxidants. The improper balance

between the reactive oxygen species produced and the antioxidant defense mechanism result in "oxidative stress", which deregulates the cellular functions leading to various pathological conditions [1]. On a daily basis, up to 5% of inhaled oxygen may be converted to reactive oxygen species (ROS) [2]. Reactive oxygen species such as superoxide anions (O_2^-), hydroxyl radical (OH^-), and reactive nitrogen species (RNS) are mainly nitric oxide (NO·), peroxynitrite (ONOO·) and nitrogen dioxide (NO2).inactivate enzymes and damage the important cellular components causing injury through covalent binding and lipid peroxidation [3]. ROS are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, crucial bimolecular such as nucleic acids, proteins and carbohydrate [4]. There is an increased evidence for the participation of ROS in the pathology of various diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases, aging, etc. [5].

Antioxidants are agents which scavenge the free radicals and prevent the damage caused by them. They can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA [6]. They play an important role in various fields such as medical field (to treat cancer, cardiovascular disorders, and chronic inflammations), cosmetics (anti- ageing process), food industries (food preservative) and others [7]. There are some synthetic antioxidant compounds such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone which are commonly used in processed foods. However, it has been suggested that these compounds have shown toxic effects like liver damage and mutagenesis [8, 9]. Therefore, attention is being directed to harness and harvest the antioxidant principles from plant materials.

Cynodon dactylon (L.) Pers. (family –Poaceae), which is commonly known as Bermuda grass and in Hindi it is known as Durva. Traditionally it is used in diabetes [10], jaundice [11], kidney problems [12] urinary disease, gastrointestinal disorder, constipation and abdominal pain [13], the whole plant is used for-diuretic, dropsy, syphilis, wound infection and piles [14]. The juice of the plant is astringent and is applied externally to fresh cuts and wounds. It is used in the treatment of catarrhal opthalmia, hysteria, epilepsy, insanity, chronic diarrhea and dysentery. The plant is folk remedy for anasarea, calcus, carbuncles, cough, hypertension, snake bites, gout and rheumatic affections [15]. The ethanolic extract of aerial parts of *C. dactylon* showed marked protection against convulsions induced by chemo convulsive agents in mice [16]. Hence the present study was designed to analyze the antioxidant capacity of *Cynodon dactylon* with different *invitro* models.

EXPERIMENTAL SECTION

Plant material

C.dactylon was collected locally in the district of Chennai, Tamail Nadu, India. The taxonomical identification and authentication was done by Dr. Sasikala Ethirajulu, Assistant Director Pharmacognosy, Siddha Central Research Institute, Arumbakkam, Chennai. A specimen was deposited in the departmental herbarium.

Preparation of Extract

The fresh green plants were first air dried (25 °C for 3 days in absence of sun light) and made into a coarse powder. It was then extracted with 80% ethanol; the resulting dark brown extract

was filtered using Whatmann No. 1 filter paper and concentrated in rotavapour under reduced pressure. The concentrated extract was lyophilized to get a powder and used for further studies.

Materials & Chemicals

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical, tannic acid, Folin- Ciocalteu reagent, ascorbic acid, quercetin, butylatedhydroxytoulene (BHT) and sodium bicarbonate were obtained from Sigma-Aldrich, USA.. All the chemicals and solvents used were of analytical grade.

Estimation of Total Phenolics content (TPC)

Total phenolic was estimated by the method of Makkar *et al.,* [17]. To a known quantity of the sample 10 ml of aqueous acetone (70%) was added and allowed to stand for 20 minutes at room temperature and then filtrated and centrifuged at 4°C for 10 minutes at 3000 rpm. The supernatant was made up to 10 ml. 0.5 ml Folin reagent and 1ml sodium carbonate were added to varying volumes of this sample and incubated at room temperature in dark for 40 minutes .Then absorbance was read at 725 nm. A calibration curve was established using varying concentration of 10% Tannic acid. The values were expressed in mg/g of sample.

Estimation of Ascorbic acid content (AAC)

The ascorbic acid content of the extract was determined according to the method of Sadasivam *et al.,* [18]. The sample was brominated and different aliquots were taken up for estimating ascorbic acid. 1ml of dinitrophenyl hydrazine (DNPH) was added followed by 100µl of thiourea. The contents were mixed thoroughly and incubated at 37 ^{o} C for 3 hours. The standard dehydroascorbic acid at a concentration of 10 -100µl was treated in a similar manner. Finally to all the tubes 7 ml of 80 % H_2SO_4 was added and the intensity of the colour was measured at 540 nm. A standard graph was plotted to determine the ascorbic acid content in the extract. The values are expressed in mg/ g of the sample.

Estimation of Total Flavonoids content (TFC)

The determination of flavonoids was performed according to the colorimetric assay of Chang *et al.,* [19]. To 1ml of varying concentrations of extract, 3 ml of methanol, 0.2ml of 1 M potassium acetate, 0.2ml of10% aluminium chloride and 5.6ml of distilled water was added and left at room temperature for 30 minutes. Absorbance of the mixture was read at415 nm using UV spectrophotometer. Calibration curve was prepared using quercetin as standard.

Total Antioxidant capacity (TAC)

To 0.1ml of varying concentration of extract, 1ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium smolybdate) was added and incubated at 95 °C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid. Prieto *et.al*., [20].

In vitro free radical scavenging activity

DPPH radical scavenging activity

The ability to scavenge the free radical DPPH was measured as decrease in absorbance at 517nm Blois M.S [21]. To 1ml of varying concentrations of extract, 1ml of DPPH (0.05mM) was added. To the final volume equal volume of alcohol was added and incubated in dark for 30 minutes.

Absorbance was recorded at 517 nm. Butylated Hydroxyl Toluene (BHT) was used as standard for comparison.

% inhibition = $[(A_0-A_1)/A_0]$ X 100, A_0-absorbance of blank, A_1- absorbance of extract

Ferrous chelating ability

The reaction mixture containing 1.0 ml of different concentrations of the extract was mixed with 3.7 ml of methanol, 0.1 ml of 2 mM ferrous chloride and 0.2 ml of 5 mM ferrozine to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. . % inhibition was calculated as above. Chelating activity was measured by the method of Huang and Kuo [22].

Superoxide anion radical scavenging assay

This assay was carried out according to the method of Liu & Chang [23], with some modification. Superoxide anion radical was generated in PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT to blue formazan. To one ml of (156 µM) nitroblueT, add 1 ml of (468 µM) of nicotinamide adenine dineucleotide and 0.5 ml of varying concentration of extract was added. To this mixture 100µl of phenazine methosulphate was added and incubated at room temperature for 5 minutes. Absorbance was read at 560 nm. % inhibition was calculated as above.

Nitric oxide scavenging assay

The method of DC Garrat [24] was followed. To 0.5 ml of varying concentration of extract, 2ml of (10 mM) sodium nitropruside, 0.5 ml of phosphate buffer saline (pH-7.4)was added and incubated at 25^0 C for 2 ½ hours .To 0.5 ml of this reaction mixture 1ml of (0.33%) sulfannilic acid was added and allowed to stand at room temperature for 5minutes. Then 1 ml of (0.1 %) naphthylene diamine chloride was added and incubated at room temperature for 30 minutes. Absorbance was read at 540 nm. % inhibition was calculated as above.

Hydroxyl radical scavenging assay

Scavenging of the hydroxyl free radical was measured by the method of W.Yu *et al.,* [25]. To 1.5 ml of varying concentration of extract, 60 µl of (1 mM) ferrous chloride, 90 µl of (0.2 M) phosphate buffer (pH 7.8) and 150 µl of (0.17 M) hydrogen peroxide was added and incubated at room temperature for 5 minutes. Absorbance was read at 560 nm. % inhibition was calculated as above.

ABTS radical scavenging assay

To 0.5 ml of varying concentration of extract, 0.3 ml of ABTS radical cation and 1.7 ml of Phosphate buffer (pH 7.4) was added. The absorbance was read at 734 nm after 10 min. incubation at room temperature. % inhibition was calculated as above. ABTS radical scavenging assay was measured by the method of Pellegrini *et al.,* [26].

Reducing Power Assay

0.5 ml of varying concentration of extract was mixed with 2.5 ml phosphate buffer and 2.5 ml of potassium ferricyanide. The mixture was incubated at 50◦C for 20 min. Aliquots of 2.5 ml trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10

min. The upper layer of solution (2.5 ml) was mixed with equal volume of distilled water, to this to mixture 0.5ml of freshly prepared ferric chloride solution was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power [2].

Hydrogen peroxide scavenging assay

Hydrogen peroxide activity of the extract was estimated by replacement titration method XY Zhang [27]. To 1ml of varying concentration of extract, 1 ml of (0.1 M) H_2O_2 and 10 ml of (2 M) H_2SO_4 and 100 µl of (3%) ammonium molybdate, 7 ml of (1.8 M) potassium iodide was added .This reaction mixture was titrated against sodium thiosulphate until the disappearance of yellow colour.

% inhibition = $[(V_0-V_1)/V_0]$ X 100,

V_0-Volume of thiosulphate used to titrate blank
V_1- Volume of thiosulphate used to titrate against the extract.

RESULTS AND DISCUSSION

Quantitative analysis of 1 gram of hydro alcoholic extract of *Cynodon dactylon* is shown in table1.Total phenolics content equivalent of tannic acid was 4.029 mg/g of extract, of total flavonoids content equivalent of quercetin was 0.17mg/g of extract and total vitamin C content equivalent of ascorbic acid was 0.114 mg/g of extract. Total antioxidant capacity equivalent of ascorbic acid was 172.39 mg/g of extract. Concentration ranging from 50-1500 µg /ml of the hydro alcoholic extract of *C. dactylon* was tested for their antioxidant activity in different *in vitro* models. It was observed that the free radical scavenged by the extract was in a dose dependant manner. Table 2&3 shows the percentage scavenging and IC_{50} values calculated for all the models. In DPPH method, the maximum scavenging activity of 78.06% was found at a concentration of 1mg/ml of the extract, its IC $_{50}$ value was270.5 µg/ml.1.5mg/ml of the extract showed 93.33% of super oxide scavenging activity and 430.06µg/ml as its IC_{50} value. The maximum scavenging activity of nitric oxide was71.28% at a concentration of 0.5 mg/ml of the extract, its IC $_{50}$ value was 115 µg/ml. TheIC_{50} value of hydroxyl radical scavenging assay 195.12µg/ml at a concentration of 0.5mg/ml and its maximum scavenging activity was 65.80%.

TABLE 1 : Quantitative analysis of phytochemical and antioxidant capacity of hydro alcoholic extract of *Cynodon dactylon*

Model	Concentration(mg/ g of extract)
TPC Tannic acid Eq	4.029
AAC Ascorbic acid Eq	0.114
TFC Quercetin Eq	0.17
TAC Ascorbic acid Eq	172.39

Although oxygen is essential for life, the generation of free radicals provokes uncontrolled reactions. This may lead to development of degenerative diseases such as diabetic complications, liver cirrhosis, nephrotoxicity, cancer etc. Reactive oxygen species such as super oxide anions, hydroxyl radicals and nitric oxide inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation.

TABLE 2 IC_{50} values of the hydro alcoholic extract of *Cynodon dactylon*

Model	IC_{50} values of *C. dactylon* (µg/ml)	Maximum scavenging concentration (mg/ml)
DPPH	270.5	1
Nitric Oxide	115	0.5
Super Oxide	430.06	1.5
Hydrogen per oxide	1350.51	1.5
Ferrous chelating	1020.86	1.5
Hydroxyl	195.12	0.5
Reducing power	632.4	1
ABTS	710	1.5

IC_{50} values denote the concentration of sample which is required to scavenge 50% of the respective free radicals. The experiments were carried out in triplicates and each value in the table was obtained by calculating the average of these.

TABLE 3 Maximum scavenging activity of hydro alcoholic extract of *Cynodon dactylon*

Model	Maximum scavenging activity (%)	Maximum scavenging concentration(mg/ml)
DPPH	78.06	1
Nitric Oxide	71.28	0.5
Super Oxide	93.33	1.5
Hydrogen per oxide	83.62	1.5
Ferrous chelating	90.88	1.5
Hydroxyl	65.80	0.5
Reducing power	67.69	1
ABTS	76.63	1.5

Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals and inhibiting the lipid peroxidation [28]. Antioxidant present in plant material act as radical scavengers, and helps in converting the radicals to less reactive species. A variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea, etc. [29]. The results of in vitro antioxidant data showed a significant free radical scavenging activity of hydro alcoholic extract of *C. dactylon* in a dose dependent manner.

The quantitative analysis of the extract showed that the extract possessed a significant amount of total phenolics than ascorbic acid and total flavonoids. Total antioxidant capacity of *C. dactylon* extract is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid [30]. The method is based on the reduction of Mo (VI) to Mo (V) by the extract at the acid pH. Total anti oxidant equivalent to vitamin C is 172.39 mg/ g of extract (Table 1).Plant phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activities [31] and are believed to prevent many degenerative diseases including cancer and atherosclerosis [32]. Total phenol content of the leaf extract expressed as Tannic acid equivalent is 4.029 mg/g.

The extract possessed significant antioxidant activity, which was capable of reducing DPPH radical to its corresponding hydrazine hence the change in colour depends on the number of electrons [33].

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and it is involved in the regulation of various physiological processes [34]. Excess concentration of NO is associated with several diseases [35, 36]. The hydro alcoholic extract of *C. dactylon* scavenged NO significantly (Table2&3) produced invitro by using sodium nitropruside. This may be due to the antioxidants present in the extract. The hydroxyl radical (OH^-) scavenging activity is measured as the % inhibition of hydroxyl radicals generated in Fenton's reaction mixture [37], these radicals may attack the sugars of DNA stand and lead to stand breakage. In the present study the extract has a maximum scavenging activity of 83.62%. It was found that at 0.5 mg/ml concentration the extract was better in scavenging NO radical than OH^- (Table2&3).

Superoxide anion is produced from molecular oxygen due to oxidative enzymes [38]. In the body it is produced by non enzymatic reactions like auto oxidation by catecholamine [39]. The scavenging activity towards the superoxide radical is measured in terms of inhibition of generation of oxygen. The extract scavenged superoxide radical significantly (93.33%) and reduced NBT to blue coloured formazan that was measured at 560 nm [40]. At 1.5mg/ml which is the maximum scavenging concentration of the extract for ABTS, super oxide and hydrogen peroxide; it is observed from table 2&3 that the extract possessed more super oxide scavenging activity comparatively. From the above studies it may be concluded that the hydro alcoholic extract of *C. dactylon* was very effective in scavenging free radicals like NO and SO significantly.

However further studies are carried out to evaluate the antioxidant potential of the extract in an *in vivo* system using Wistar rats as animal model.

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