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Radical scavenging and antioxidant activity of ethylacetate fraction of *Plectranthus aromaticus* leaves

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ABSTRACT

Free radical damages have been implicated for more than hundred diseases including diabetes mellitus, arthritis, cancer, ageing etc. In treatment of these diseases, antioxidant therapy has gained an utmost importance. Current research is now directed towards finding of naturally occurring antioxidant of plant origin. In Indian system of medicine *Plectranthus aromaticus* is an important medicinal plant and its leaves are often eaten raw with bread and butter, and decoction of its leaves is administered in the cases of chronic cough and asthma. To understand the pharmacological actions, *in vitro* antioxidant activity of ethyl acetate fraction of *P. aromaticus* was investigated for scavenging super oxide anion radicals, hydroxyl radicals, nitric oxide radicals and hydrogen peroxide, metal chelation and total antioxidant power. The fraction was also studied for lipid peroxidation assay using sheep liver homogenate. In all these tests, a significant correlation existed between concentrations of extract and percentage of inhibition of free radicals, metal chelation, total antioxidant power and inhibition of lipid peroxidation.

Key words: Free radicals, antioxidant therapy, *Plectranthus aromaticus*, lipid peroxidation.

INTRODUCTION

Majority of the disorders are mainly linked to oxidative stress due to free radicals (Gutteridgde, 1995). Free radicals are fundamental to many biochemical processes and represent an essential part of aerobic life and metabolism (Tiwari, 2001). The most common reactive oxygen species (ROS) include superoxide ($0_2^{\cdot}$) anion, hydrogen peroxide (H_20_2), peroxyl ($ROO^{\cdot}$) radicals and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^{\cdot}$). ROS have been implicated in over a hundreds of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (Joyce, 1987). In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now focused towards finding of natural antioxidants from plant origin.

Antioxidants have been reported to prevent oxidative damage by free radical and ROS and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers (Buyukokuroglu *et al*., 2001; Shahidi and Wanasundara, 1992). Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant

activities, no side effects and economic viability (Auudy *et al*., 2003). Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties (Havsteen , 1983; Boyer *et al*., 1988; Miller , 1996).

Plectranthus aromaticus syn Coleus aromaticus (*Laminaceae*), popularly called as India borage and several medicinal properties have been attributed to this plant in the Indian system of medicine. Traditionally, it is often used for medicinal purposes as part of diet. The leaves of the green type country borage are often eaten raw with bread and butter. It is also used for seasoning meat dishes and food products (Uhof, 1959), while a decoction of its leaves is administered in the cases of chronic cough and asthma (CSIR, 1992). It is considered to be antispasmodic, stimulant and stomachic and is used for the treatment of headache, fever, epilepsy and dyspepsia (Morton, 1992; Khory and Katrak, 1999). In India, it is widely used for various illnesses, including kidney stones (Vaidyaratnam, 1995). It is scientifically evaluated for its antibacterial (Timer *et al*., 1992) and antioxidant activity (Padma *et al*., 1998).

There fore, the objectives of the present study is to investigate the in vitro antioxidant activity of *P. aromaticus* in terms of its ability to scavenge free radicals like superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide radicals, metal chelation and inhibition of lipid peroxidation.

MATERIAL AND METHODS

All the chemicals used in the present study are of analytical grade and obtained from local suppliers. The leaves of *Plectranthus aromaticus* are obtained from regional agricultural research station, Chinthapalli, Andhra Pradesh, India. They are authenticated by the department of Botany, Andhra University.

Extraction and fractionation

The leaves were thoroughly cleaned, shade dried and powdered in a mechanical grinder. The sterilized powder (50g) of the leaves is extracted with 250ml of ethanol using a Soxhlet extractor for 48 hrs. The extract is filtered using Whatman (No1) filter paper and then evaporated to dryness in vacuum. The ethanol extract (2g) was subjected to fractionation by column chromatography on silica gel (60-120 mesh, 0.5x25 cm) and eluted with Hexane (20ml), ethyl acetate (20ml). The fractions are analyzed by TLC on silica gel G plates (20x5cm) using the solvent system hexane-ethyl acetate (95:5). The spots were visualized by spraying with Vanillin/Sulfuric acid reagent. The fractions (F11-F20) obtained with ethyl acetate showed antioxidant power by FRAP method, which were pooled, concentrated (500mg) and further used for assaying various radical scavenging activities. The percentage of inhibition of radicals was calculated by comparing the results of the test with those of the control. Percentage of inhibition = A_o–A x 100/ A_o. There fore, A_o is Absorbance of control and A is Absorbance of test sample. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of free radicals.

Total antioxidant power

Total antioxidant power was determined by the modified FRAP (Ferric chloride reducing ability of plasma) method of Benzie and Strain (1996). In this assay, the FRAP reagent is prepared by adding 2, 4, 6-tripyridyl-s-triazine (TPTZ) and ferric chloride forming the Fe^{+3}-TPTZ complex. In presence of an antioxidant, the Fe^{+3}-TPTZ complex is reduced to Fe^{+2}-TPTZ complex which gives an intense blue color with maximum absorption at 595nm. The calibration curve was prepared using $FeSO_4$ with concentrations ranging from 0-1mM. In this assay, ethyl acetate fraction of *P. aromaticus*, BHT and Quercetin are used at concentration of 0.1mg%. The results are expressed as Ascorbic acid Equivalent Antioxidant Capacity (AEAC) in terms of mM.

DPPH radical scavenging activity

DPPH scavenging activity was measured by the method of Koleva *et al*., (2002). To 1.0 ml of an ethanolic solution of DPPH (0.3mM), 2.5 ml of test extract/ Butylated hydroxy toluene (BHT)/ Quercetin dissolved in ethanol were added. The test samples are prepared in different concentrations (100-1000µg). For control, test sample is replaced by ethanol. The solutions were incubated at 37°C for 30 min, absorbance measured at 517 nm using Hitachi UV visible spectrophotometer.

Hydroxyl radical scavenging activity

The ability of the sample to inhibit hydroxyl radical mediated peroxidation was carried out according to method of Kunchandy and Rao (1990) with some adaptations. The reaction mixture contained 100ml of different concentration of ethyl acetate fraction of *P. aromaticus* (100-1000 µg), 500µl of 0.6mM deoxyribose in phosphate buffer (25mM, pH 7.4), 200µl of premixed 0.02mM ferrous ammonium sulfate and 0.02 mM EDTA (1:1 v/v) solution, 100µl of ascorbic acid (0.6mM) and 100ml of H_2O_2 (0.85mM). After 15 min of incubation at 37°C, a 1.5 ml of 2.8% cold TCA and 1.0ml of TBA were added. The samples were vortexed and heated in a water bath at 50°C for 15 min. The absorbance was determined at 532nm.

Super oxide radical scavenging activity

The super oxide scavenging activity was measured by Beauchamp and Fridovich method (1971) with some modifications. Superoxide anions were generated in a non-enzymatic hydroxyl amine (HA) – EDTA system though the reaction of HA, EDTA and oxygen. It was assayed by the reduction of nitroblue tetrazolium. In these experiments, the super oxide anion was generated in a reaction mixture containing 1.0 ml of sodium carbonate (125mM), 0.4ml NBT(25µM) and 0.2ml of EDTA (0.1mM). The reaction was initiated by adding 0.4ml of hydroxyl amine (0.1mM) and 0.5ml of different concentrations of the extracts (100-1000µg). Control was set up without plant extract. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The control was simultaneously run without plant extract.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the extract was estimated by titration method of Zhang, (2000). Aliquot of 1.0ml of 0.1mM H_2O_2 and 1.0ml of various concentrations (100-1000µg) of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10ml of 2M H_2SO_4 and 0.7 ml of 1.8M KI. The mixed solution was titrated with 5.09mM $Na_2S_2O_3$ until yellow color disappeared.

Nitric oxide scavenging activity

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat, (1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide ions, which can be determined by the use of Griess illosvoy reaction. 2 ml of 10mM sodium nitroprusside in 0.5ml phosphate buffer saline (pH 7.4) was mixed with 0.5ml of extract at various concentrations (100-1000µg) and the mixture incubated at 25°C for 150min. From the incubation mixture 0.5ml solution was taken out and added to 1.0ml of sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally 1.0 ml of naphthylenediamine dihyrochloride (0.1%w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540nm was measured with a spectrophotometer.

Fe^{2+} chelating activity

The chelating activity of the ethyl acetate fraction of *P. aromaticus* for ferrous ions was measured according to the method of Dinis *et al.*, (1994). To 0.5 ml of extract, 1.6ml of distilled water and 0.05ml of $FeCl_2$ (2mM) was added. After 30s, 0.1 ml of ferrozine (5mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562nm.

In vitro inhibition of lipid peroxidation

Lipid peroxidation induced by $FeSO_4$-ascorbate system in sheep liver homogenate by the method of Bishayee and Balasubramaniyam (1971) was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al.*,(1979) The reaction mixture contained 0.1ml of sheep liver homogenate (25%) in Tris-HCl buffer (20mM, pH 7.0; KCl (30mM); $FeSO_4$ (NH_4) $SO_4.7H_2O$ (0.06 mM) and various concentrations of ethyl acetate fraction of *P. aromaticus* in a final volume of 0.5ml. The reaction mixture was incubated at 37°C for 1h. After the incubation, 0.4ml was removed and treated with 0.2ml sodium dodecyl sulphate (SDS) (8.1%), 1.5ml thiobarbituric acid (TBA) (0.8%) and 1.5ml of trichloroacetic acid (20%, pH 3.5). The total volume was made up to 4.0ml with distilled water and then kept in a water bath at 95°C for 1h. After cooling, 1.0ml of distilled water and 5.0ml of n-butanol and pyridine mixture (15:1) were added to the reaction mixture, shaken

vigorously and centrifuged at 4000g for 10 min. The butanol pyridine layer was removed and its absorbance was measured at 532 nm. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of the extract with that of the control. Quercetin and BHT were used as standard.

Statistical analysis

All treatments were performed in five times and each data point in the results is the mean of five replicates. All experiments were repeated at least twice. The statistical significance of a treatment effect was evaluated by student's t-test and the values were expressed as mean ± SD. Probability limit was set at $p<0.05$.

RESULTS AND DISCUSSION

Ethyl acetate fraction of *P. aromaticus* leaves was tested for its total antioxidant power by the FRAP assay. Table 1. presents data on AEAC values of the ethyl acetate faction of *P. aromaticus* and standard antioxidants such as BHT and Quercetin at concentration of 0.1%. In this assay ethyl acetate fraction of *P. aromaticus* possessed the highest AEAC value of 0.057mM, followed by BHT and Quercetin with 0.049 and 0.047mM respectively.

Table 1. Total antioxidant power of ethyl acetate fraction of *P. aromaticus* leaves

Sample	Total antioxidant power (AEAC, mM)
Ethyl acetate fraction	0.057±0.002
BHT	0.049±0.001
Quercetin	0.047±0.001

Values are means ± SD (n=5). Data expressed as total antioxidant activity as ascorbic acid equivalent antioxidant capacity (AEAC, mM).

Ethyl acetate fraction of *P. aromaticus* leaves was tested for radical scavenging activity in different *in vitro* models. In all radical scavenging assays, the test sample showed radical scavenging activity in a concentration dependent manner up to the given concentration in the model studied. In DPPH model, maximum percentage of inhibition was observed (Table 2) with ethyl acetate fraction of *P. aromaticus* (89.31) followed by BHT (76.22) and Quercetin (61.52) at concentration of 0.1mg per ml respectively. IC_{50} values of *P. aromaticus*, BHT and Quercetin are 325, 525, 650 µg/ml respectively.

Table 2: DPPH radical scavenging activity of ethyl acetate fraction of *P. aromaticus* leaves.

Concentration (mg/ml)	Percentage of inhibition		
	Ethyl acetate fraction	Quercetin	BHT
100	32.25±0.25	21.02±0.28	23.11±0.31
200	41.13±0.34	28.12±0.34	30.13±0.24
300	49.05±0.19	32.15±0.31	37.14±0.35
400	56.12±0.22	37.22±.027	42.10±0.26
500	67.43±0.31	43.24±0.22	49.11±0.28
600	74.35±0.42	48.32±0.31	53.14±0.29
700	79.65±0.24	51.36±0.24	59.13±0.34
800	93.52±0.19	56.42±0.25	65.22±0.36
900	86.45±0.42	59.45±0.28	71.21±0.41
1000	89.31±0.33	61.52±0.32	76.22±0.46
IC_{50}	325	650	525

Values are means ± SD (n=5)

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Ohkawa *et al*., 1979). The superoxide anion radical scavenging activity of the ethyl acetate fraction of *P. aromaticus* is shown in table 2. The super oxide scavenging was increased with the increase of concentrations. The half inhibition concentration (IC_{50}) of *P. aromaticus*, BHT and Quercetin are 375, 580, 675 µg/ml respectively. These results suggest that *P. aromaticus* had potent super oxide radical scavenging effect.

Hydroxyl radical is very reactive and can be generated in biological cells through Fenton reaction. Table 4. showed that ethyl acetate fraction of *P. aromaticus* exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC_{50} of *P. aromaticus* was 275 µg/ml, where as BHT and Quercetin were 525 and 625 µg/ml.

Nitric oxide is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodialation and antimicrobial and antitumor activities (Hagerman *et al*., 1998). Ethyl acetate fraction of *P. aromaticus* was moderately inhibited nitric oxide in dose dependent manner (Fig.1) with the IC_{50} of 119 µg/ml.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Miller *et al*., 1993). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. As shown in Fig 2, ethyl acetate fraction of *P. aromaticus* demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC_{50} value of 110 µg per ml, which is lower than BHT and Quercetin.

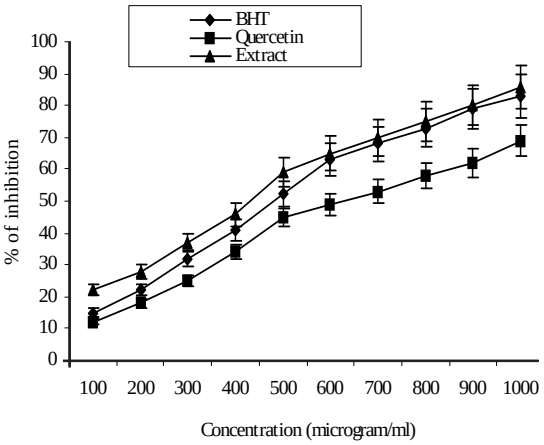

Fig. 1: Nitric oxide radical scavenging activity of ethyl acetate fraction of *P .aromaticus* leaves.

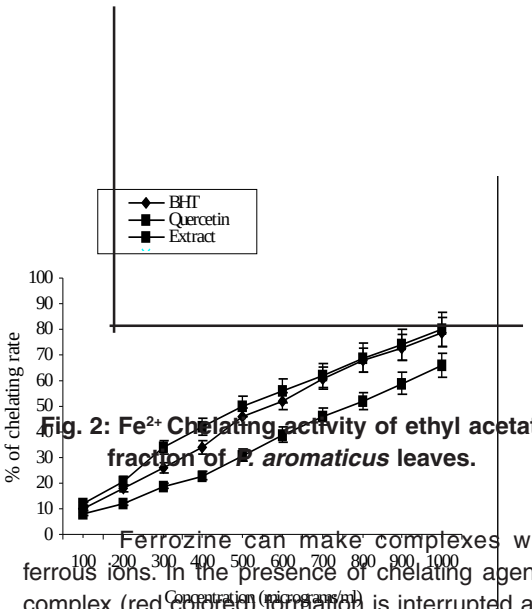

Fig. 2: Fe^{2+} Chelating activity of ethyl acetate fraction of *P. aromaticus* leaves.

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine – Fe^{2+} complex is interrupted in the presence of ethyl acetate fraction of *P. aromaticus*, indicating the chelating activity with an IC_{50} of 100 µg per ml. However IC_{50} of BHT and Quercetin are 110 and 158 µg per ml (Fig 3).

Initiation of the lipid peroxidation by ferrous ion takes place through hydroxyl radical by Fenton

reaction. Fig 4. shows that the acetyl acetate fraction of *P. aromaticus* inhibited $FeSO_4$ induced lipid peroxidation in sheep liver homogenate in a dose dependent manner. The inhibition could be caused by scavenging the hydroxyl radical or the super oxide radicals or by changing the Fe^{3+}/Fe^{2+} or by chelating the iron itself. Iron catalyzes the generation of hydroxyl radical from hydrogen peroxide and super oxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydro-peroixdes are produced (Valentao *et al*., 2002). Lipid hydro-peroxide can be decomposed to produce alkoxy and peroxy radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and ageing related diseases (Reimersma *et al*., 2000). Thus the decrease in the MDA level in sheep lever

Fig. 3: Hydrogen peroxide scavenging activity of ethyl acetate fraction of *P. aromaticus* leaves.

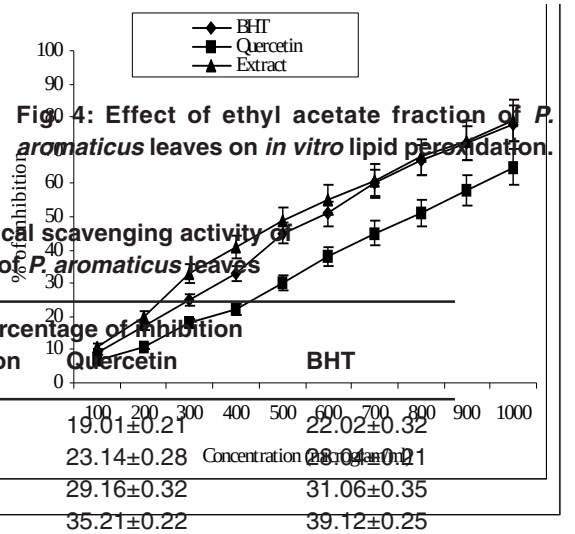

Fig. 4: Effect of ethyl acetate fraction of *P. aromaticus* leaves on *in vitro* lipid peroxidation.

Table 3: Super oxide radical scavenging activity of ethyl acetate fraction of *P. aromaticus* leaves

Concentration (µg/ml)	Percentage of inhibition		
	Ethyl acetate fraction	Quercetin	BHT
100	31.15±0.21	19.01±0.21	22.02±0.32
200	38.16±0.32	23.14±0.28	28.04±0.21
300	43.25±0.17	29.16±0.32	31.06±0.35
400	54.17±0.21	35.21±0.22	39.12±0.25
500	63.42±0.28	41.22±0.27	44.08±0.28
600	69.38±0.36	47.31±0.31	51.11±0.26
700	74.45±0.26	52.32±0.23	58.10±0.32
800	80.51±0.21	57.41±0.22	62.14±0.31
900	82.45±0.19	62.42±0.27	70.15±0.38
1000	85.71±0.32	71.52±0.32	82.12±0.42
IC_{50}	375	675	580

Values are means ± SD (n=5)

Table 4: Hydroxyl radical scavenging activity of ethyl acetate fraction of *P. aromaticus* leaves

Concentration (µg/ml)	Percentage of inhibition		
	Ethyl acetate fraction	Quercetin	BHT
100	37.14±0.18	20.08±0.31	24.09±0.30
200	42.15±0.16	26.15±0.18	29.01±0.27
300	51.18±0.20	32.18±0.31	36.08±0.31
400	59.41±0.27	37.31±0.21	41.14±0.21
500	65.44±0.21	42.21±0.22	48.02±0.22
600	71.35±0.31	49.01±0.41	56.12±0.20
700	76.41±0.22	55.31±0.20	61.20±0.31
800	81.52±0.21	61.41±0.21	68.10±0.12
900	87.42±0.19	67.40±0.20	72.65±0.30
1000	93.70±0.32	73.50±0.31	84.14±0.12
IC_{50}	275	625	525

Values are means ± SD (n=5)

homogenate with increase in the concentration of the ethyl acetate fraction of *P. aromaticus* indicates its role as a potent antioxidant.

In conclusion, the results of the present study show that the ethyl acetate fraction of *P. aromaticus* exhibits significant antioxidant activity through the scavenging of different free radicals such as super oxide , hydroxyl radical, hydrogen peroxide, nitric oxide and also iron chelation, which participate in various pathophysiology of diseases including ageing. Overall, the plant extract is a source of natural antioxidants that can be important in disease prevention, health preservation and promotion of longevity of life. Further phytological analysis is required to identify the chemical constituents responsible for its antioxidant activity.

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